



## ARTICLE

# Characterization of CYP3A pharmacogenetic variation in American Indian and Alaska Native communities, targeting CYP3A4\*1G allele function

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## Abstract

The frequencies of genetic variants in the *CYP3A4* and *CYP3A5* genes differ greatly across global populations, leading to profound differences in the metabolic activity of these enzymes and resulting drug metabolism rates, with important consequences for therapeutic safety and efficacy. Yet, the impact of genetic variants on enzyme activity are incompletely described, particularly in American Indian and Alaska Native (AIAN) populations. To characterize genetic variation in *CYP3A4* and *CYP3A5* and its effect on enzyme activity, we partnered with AIAN people living in two regions of Alaska: Yup'ik Alaska Native people living in the Yukon-Kuskokwim Delta region of rural southwest Alaska and AIAN people receiving care at the Southcentral Foundation in Anchorage, Alaska. We identified low frequencies of novel and known variation in *CYP3A4* and *CYP3A5*, including low frequencies of the *CYP3A4\*1G* and *CYP3A5\*1* variants, and linkage disequilibrium patterns that differed from those we previously identified in an American Indian population in western Montana. We also identified increased activity of the *CYP3A4\*1G* allele in vitro and in vivo. We demonstrated that the *CYP3A4\*1G* allele confers increased protein content in human lymphoblastoid cells and both increased protein content and increased activity in

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human liver microsomes. We confirmed enhanced CYP3A4-mediated 4 $\beta$ -vitamin D hydroxylation activity in Yup'ik people with the *CYP3A4\*1G* allele. AIAN people in Alaska and Montana who carry the *CYP3A4\*1G* allele—coupled with low frequency of the functional *CYP3A5\*1* variant—may metabolize CYP3A substrates more rapidly than people with the reference *CYP3A4* allele.

## INTRODUCTION

The human cytochrome P450 3A enzymes, CYP3A4 and CYP3A5, metabolize many xenobiotics and dominate the clearance of about one-third of all medications. The frequencies of genetic variants in the *CYP3A4* and *CYP3A5* genes differ exceptionally across global populations, leading to profound differences in the metabolic activity of these enzymes and resulting drug metabolism rates, with important consequences for therapeutic safety and efficacy.<sup>1–3</sup> Understanding *CYP3A4* and *CYP3A5* genetic variation, and the resulting effects on enzyme activity across diverse populations and allele combinations, is essential for improving the equity and utility of preemptive pharmacogenetic testing.

Compared with the reference *CYP3A4\*1A* allele (herein referred to as *CYP3A4\*1*), a number of single nucleotide variants (SNVs) have been associated with a change in the metabolic clearance of CYP3A drug substrates.<sup>4</sup> SNVs that have received the most research attention are *CYP3A4\*1B*, *CYP3A4\*1G*, and *CYP3A4\*22*, which arise from noncoding variants thought to affect gene transcription or the stability of mRNA.<sup>5–7</sup> Whereas the *CYP3A4\*22* allele has been associated consistently with reduced enzyme activity and reduced clearance of substrates, such as tacrolimus, simvastatin, cyclosporine, and atorvastatin, *CYP3A4\*1B* and *CYP3A4\*1G* remain poorly characterized, each having been associated with both decreased and increased enzyme activity.<sup>6–30</sup> Most variation in *CYP3A5* is associated with loss-of-function alleles, conferred primarily by three alleles, including a common variant (*CYP3A5\*3*) that results in aberrant mRNA splicing. The other two, *CYP3A5\*6* and *CYP3A5\*7*, are splicing defect and frameshift variants, respectively, and are seen more often in populations of African descent.<sup>31,32</sup> The frequency of the functional *CYP3A5\*1* reference allele varies widely between populations: ~7% in Europeans and 47% in Sub-Saharan African populations.<sup>33</sup>

In prior work, we identified a unique linkage disequilibrium pattern with the *CYP3A4\*1G* allele in participants from the Confederated Salish and Kootenai Tribes (CSKTs) in western Montana<sup>34</sup>; we observed a relatively high frequency of *CYP3A4\*1G*, but a low level of linkage disequilibrium between *CYP3A4\*1G* and *CYP3A5\*1*, the functional allele of *CYP3A5*. These findings are unique because CYP3A4 protein expressed as a consequence of the *CYP3A4\*1G* variant exists in the absence of functional CYP3A5 protein for a majority of CSKT people compared with the minority of people in many world

### Study highlights

#### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Alleles in *CYP3A4* and *CYP3A5* are highly variable in populations globally, but have not been well-characterized in American Indian and Alaska Native (AIAN) populations. The functional effects of some variants are not well known, including the *CYP3A4\*1G* allele, which we identified in unique linkage disequilibrium patterns in prior work with an American Indian population in Montana.

#### WHAT QUESTION DID THIS STUDY ADDRESS?

This study characterizes the *CYP3A4\*1G* allele using in vitro and in vivo models. It also improves our understanding of genetic variation across AIAN populations in North America.

#### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

We show increased activity of the *CYP3A4\*1G* allele, and identify low frequencies of *CYP3A4* and *CYP3A5* variants, including *CYP3A4\*1G* and functional *CYP3A5\*1*, in Alaska Native people.

#### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Inclusion of diverse populations in estimating diplo-type frequencies and their function is important for ensuring equitable access to the benefits of personalized medicine for all. We showed that people with the *CYP3A4\*1G* allele may require dose escalation of CYP3A4-metabolized medications due to increased activity of the CYP3A4 enzyme.

populations. The goal of this study was to assess the functional effects of the *CYP3A4\*1G* allele in both in vitro and in vivo models. Additionally, we also sought to characterize genetic variation in *CYP3A4* and *CYP3A5* genes in other American Indian and Alaska Native (AIAN) populations. Through collaborative partnerships with the Southcentral Foundation (SCF), a tribal health organization in Anchorage, AK, serving AIAN people, and with Yup'ik Alaska Native people from

the Yukon-Kuskokwim (YK) Delta, we identified novel variation and population allele frequencies within the *CYP3A4* and *CYP3A5* genes in AIAN populations living in different regions of Alaska.

## METHODS

### Institutional review board approvals and research settings

The Alaska Area Institutional Review Board (AAIRB) and SCF tribal review boards, Yukon-Kuskokwim Health Corporation (YKHC) Executive Board of Directors, and institutional review boards from the University of Alaska Fairbanks and University of Washington approved this research. Research questions were developed in partnership with tribal representatives through community-based participatory research; the partnerships with SCF, YKHC, and CSKT have been described previously in detail.<sup>35,36</sup>

### *CYP3A4* and *CYP3A5* genetic variant discovery and frequency assessment

A convenience sample of 363 SCF and 350 YK participants, aged greater than or equal to 18 years contributed DNA. To discover any common novel SNVs, we resequenced *CYP3A4* and *CYP3A5* in a subset of these participants: 188 and 94 from the SCF and YK cohorts, respectively (referred to as the resequencing study populations). Participants from the YK cohort were confirmed to be unrelated through pedigree analysis. The methods for blood collection, buffy coat isolation, and DNA extraction of samples are described previously.<sup>37</sup> Exons and adjacent intronic regions of *CYP3A4* and *CYP3A5* were resequenced, as described previously.<sup>34</sup>

Next, we assessed population frequencies of the SNVs. Among all participants within the SCF ( $n = 363$ ) and YK ( $n = 350$ ) cohorts (referred to as the genotyping study populations), we genotyped named star alleles, previously identified nonsynonymous variants, and previously unreported nonsynonymous variants that we identified during the resequencing phase. As a result, we genotyped 8 SNVs in *CYP3A4* and 8 SNVs in *CYP3A5* using the Fluidigm Specific Target Amplification protocol and Genotyping assays (South San Francisco, CA) as described previously.<sup>37,38</sup> DNA samples with call rates below 95% across all SNVs were removed from further analysis. Of samples initially selected for genotyping, 17 from the SCF (4.5%) were excluded; all samples from the YK met quality thresholds. Concordance was 100% between samples assessed by both resequencing and genotyping.

### *CYP3A4* protein content in human lymphoblastoid cell lines

Human lymphoblastoid cell lines (LCLs) from the Peruvian in Lima, Peru (PEL) population in the 1000 Genomes Collection (Coriell Institute, Camden, NJ) varied by *CYP3A4* diplotypes *CYP3A4\*1/\*1* ( $n = 4$ ), *CYP3A4\*1/\*1G* ( $n = 4$ ), and *CYP3A4\*1G/\*1G* ( $n = 4$ ). LCLs from the PEL population were chosen because—similar to our observations in the CSKT population—there is low linkage disequilibrium between *CYP3A4\*1G* and *CYP3A5\*1*. Therefore, we could select LCL samples with desired *CYP3A4\*1G* diplotypes that were also all homozygous *CYP3A5\*3/\*3*, which eliminated contribution from *CYP3A5*. The *CYP3A4\*1B* and *CYP3A4\*22* variants were also not present in these samples. LCLs were grown according to Coriell's recommended protocols. Briefly, cells were cultured at 37°C in RPMI 1640 media (with 2 mM L-gln, 15% FBS, and 1% penicillin/streptomycin) and grown at 37°C in the presence of 5% CO<sub>2</sub>. Approximately  $1 \times 10^6$  cells were pelleted, lysed (40 mM Tris, 120 mM NaCl, 1% Triton X-100, 0.1% SDS, pH 7.3, and protease inhibitors), and lysates collected and stored at -80°C. Protein concentration of cell lysates was determined using Bio-Rad Protein Assay Dye according to manufacturer's protocol using BSA as a standard.

*CYP3A4* protein content in LCLs was measured using a standard Bio-Rad (Hercules, CA) immunoblot protocol according to manufacturer's protocol with SDS-PAGE Mini-PROTEAN TGX gels (Bio-Rad). Recombinant *CYP3A4* enzyme from baculovirus-infected insect cells was used as a positive control (BD Gentest; Corning, Corning, NY). *CYP3A4* was detected with primary antibody, *CYP3A4* Mouse MaxPab (Abnova, Taipei City, Taiwan), diluted (1:1,000) in blocking buffer (Tris-buffered saline +0.2% Tween +7% nonfat milk), and secondary antibody, HRP-conjugated anti-mouse (Thermo Fisher) diluted (1:100,000) in blocking buffer +1% goat. Endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was measured with HRP-conjugated anti-human GAPDH antibody (Thermo Fisher) diluted (1:200,000) in blocking buffer +1% goat. The membrane was developed with SuperSignal West Femto kit, according to its manufacturer's protocol (Thermo Fisher). The membrane was visualized using a FluorChem M system (Protein Simple, San Jose, CA). *CYP3A4* protein was quantitated relative to GAPDH in each lane.

### DNA isolation, *CYP3A4* and *CYP3A5* sequencing, and mRNA and protein quantitation in human liver microsomes

Human liver tissue samples ( $n = 319$ ) were obtained from the University of Washington Human Liver Bank ( $n = 59$ ) and the St. Jude Liver Resource at St. Jude Children's Research Hospital ( $n = 260$ ). Preparation of human liver

microsome (HLM) and quantitation of total protein were described previously.<sup>39</sup> Methods for DNA isolation, quantitation of aldo-keto reductase 1D1 (*AKR1D1*) mRNA content, and quantitation of CYP3A4 and cytochrome P450 oxidoreductase (POR) protein content were as described previously.<sup>39–43</sup> *CYP3A4* and *CYP3A5* sequencing was performed using the PGRNseq platform (version 1.1), which captures all exons as well as 2 kb of upstream and 1 kb of downstream sequence.<sup>44</sup> *CYP3A4* and *CYP3A5* alleles are defined according to the Pharmacogene Variation Consortium.<sup>4</sup>

## CYP3A4 activity in human liver microsomes

CYP3A4 activity in HLMs was determined with the P450-Glo CYP3A4 Assay System (Promega, Madison, WI) according to manufacturer's specifications with the CYP3A4-specific substrate, Luciferin-IPA, which does not measure any CYP3A5 activity. Microsomal incubation conditions were optimized using pooled HLMs (Xenotech, Lenexa, KS) to ensure experiments were in the linear range of substrate concentration, total HLM protein content, and incubation time (data not shown). Five  $\mu\text{g}$  of total HLM protein were preincubated with 3  $\mu\text{M}$  P450-Glo CYP3A4 substrate (Luciferin-IPA) and 100 mM potassium phosphate buffer for 10 min in a water bath at 37°C. An equal volume of 2 mM NADPH was added to initiate the reaction and HLMs were incubated for 10 min in a water bath at 37°C. An equal volume of Luciferin Detection Reagent was added and incubated for 15–20 min at room temperature. All incubations were completed in triplicate. Pooled HLMs were used as a positive control in every incubation set. Luminescence was detected on a SynergyMX microplate reader (Biotek, Winooski, VT).

## Endogenous CYP3A4-mediated vitamin D hydroxylation

We recruited a convenience sample of 743 self-identified Yup'ik participants, greater than or equal to 14 years of age from the YK Delta, and selected 526 who were unrelated to the third degree to reduce nonindependence, as described previously.<sup>45</sup> From this subset, 514 participants were successfully genotyped for *CYP3A4\*1G* using the Fluidigm genotyping platform described above. These participants included the 350 that also participated in the gene variant discovery and population allele frequency studies, described above. The unrelated subset was representative of the overall dataset.<sup>45</sup> We collected serum samples and quantified serum 4 $\beta$ ,25-dihydroxyvitamin D<sub>3</sub> (4 $\beta$ ,25[OH]<sub>2</sub>D<sub>3</sub>) and 25-hydroxyvitamin D<sub>3</sub> (25[OH]D<sub>3</sub>), as described previously.<sup>46</sup> Adjusting for gender, we compared serum 4 $\beta$ ,25(OH)<sub>2</sub>D<sub>3</sub>/25(OH)D<sub>3</sub> between participants with at least one *CYP3A4\*1G* allele and those with no copies of the

allele because we only identified 3 participants homozygous for the *CYP3A4\*1G* allele.

## Statistical analysis

We performed all analyses in R programming language. We used pedigrees and the best linear unbiased estimator,<sup>47</sup> as described in ref. 45, to calculate allele frequencies and confidence intervals for the SCF and YK cohorts. We tested all SNVs for deviations from Hardy–Weinberg equilibrium using a  $\chi^2$  test, with a significance level of 0.05. A *p* value less than 0.05 (*p* < 0.05) was considered to be statistically significant for all analyses.

The association of the *CYP3A4\*1G* diplotype and CYP3A4 protein content in LCLs was estimated using one-way analysis of variance with GraphPad Prism 7.0 software (GraphPad, San Diego, CA). CYP3A4 protein content and activity in HLMs were tested for skewness and kurtosis, and log transformed to improve normal distribution. We used multivariable linear regression to assess the association of *CYP3A4\*1G* diplotype and CYP3A4 protein content and activity in HLMs. All analyses of HLMs were adjusted for site of sample collection, which has been consistently associated with differences in outcomes.<sup>39,40,43</sup> Primary analysis used linear regression, adjusting for site of sample collection, to assess the association of the *CYP3A4\*1G/\*1G* diplotype on log transformed CYP3A4 protein content and activity, compared with livers that did not have this diplotype. Secondary analysis using multiple linear regression was used to assess the influence of other potential covariates on log transformed CYP3A4 protein and activity, including age (continuous), sex (binary: male vs. female), POR protein content (continuous), presence of the *CYP3A4\*22* allele (binary: presence or absence), and *AKR1D1* mRNA content (continuous). Covariates with significant unadjusted associations with CYP3A4 protein content and activity in the primary analysis were kept in the final multivariable model to determine the association between *CYP3A4\*1G* diplotype and both log CYP3A4 protein content and activity. We used an unpaired *t*-test to compare serum 4 $\beta$ ,25(OH)<sub>2</sub>D<sub>3</sub>/25(OH)D<sub>3</sub> between participants with at least one *CYP3A4\*1G* allele and those with no copies of the allele.

## RESULTS

### *CYP3A4* and *CYP3A5* genetic variant discovery and frequency assessment

#### Population demographics

Table 1 summarizes the demographics for the SCF and YK participants included in the cohorts for the DNA genotyping study and the YK participants in the vitamin D hydroxylation

study. All participants self-identified as Yup'ik in the YK cohort or as AIAN in the SCF cohort. Donor demographics for the HLM study are also summarized in Table 1.

## Resequencing for SNV discovery

Tables S1 and S2 present the SNVs—known and previously unreported—identified in resequencing *CYP3A4* and *CYP3A5*, respectively, from a convenience sample cohort of SCF ( $n = 188$ ) and YK ( $n = 94$ ) participants from the genotyping study. In addition to previously identified SNVs, we identified two variants in *CYP3A5* that had not been described previously: a nonsynonymous variant at position chr7:99245986 resulting in a proline to leucine change at amino acid 484 that was identified in the SCF cohort and a

deletion at chr7:99247737 resulting in a frameshift at amino acid 458 that was identified in the YK cohort, and has since been assigned rs547253411.

## Genotyping for assessing population frequencies

All SNVs were in Hardy–Weinberg equilibrium in a larger cohort of SCF ( $n = 363$ ) and YK ( $n = 350$ ) participants. Table 2 presents the frequencies of the novel and known important alleles that were genotyped in the SCF and YK cohorts in relation to other populations around the globe.

The *CYP3A4\*1G* allele was infrequent in the SCF cohort (12.7%) relative to other populations and the frequency in the YK cohort (4.0%) was the lowest reported in any population to date. The nonfunctional *CYP3A5\*3* allele was found at high frequencies in both the SCF (89.8%) and YK (95.9%) cohorts. The linkage disequilibrium between *CYP3A4\*1G* and *CYP3A5\*1* was  $r^2 = 0.55$  in the SCF cohort and  $r^2 = 0.90$  in the YK cohort (Figure S1). Importantly, the *CYP3A5\*3/\*3* diplotype is associated with very low expression of CYP3A5 enzyme compared with either the *CYP3A5\*1/\*1* and *CYP3A5\*1/\*3* diplotypes. Based on these phenotypes, only 19.6% of the SCF cohort and 8.9% of the YK cohort are expected to have functional CYP3A5 activity (i.e., people with at least one copy of the *CYP3A5\*1* allele).

**TABLE 1** Demographics of study populations

Study populations	
SCF genotyping ( $n = 363$ )	
Sex, $n$	
Male	127 (35.0%)
Female	236 (65.0%)
YK genotyping ( $n = 350$ )	
Sex, $n$	
Male	163 (46.6%)
Female	187 (53.4%)
HLM set ( $n = 319$ )	
Age, years <sup>a</sup>	
Mean $\pm$ SD	39.6 $\pm$ 22.2
Range	0–87
Sex, $n$	
Male	185 (58.0%)
Female	129 (40.4%)
Unknown	5 (1.6%)
Race/ethnicity, $n$	
European	288 (90.3%)
African	5 (1.6%)
Asian	1 (0.3%)
Hispanic	1 (0.3%)
Unknown	24 (7.5%)
YK vitamin D hydroxylation ( $n = 526$ )	
Sex, $n$	
Male	282 (53.6%)
Female	244 (46.4%)

Abbreviations: HLM: human liver microsome; SCF, Southcentral Foundation; YK, Yukon Kuskokwim.

<sup>a</sup>Age was recorded in years for all subjects and an age of zero was given to donors under 1 year old; more granular data (e.g., weeks, months) were not available.

## CYP3A4 protein content in human lymphoblastoid cell lines

*CYP3A4\*1G* was significantly associated with increased CYP3A4 protein content, normalized to endogenous control GAPDH ( $p < 0001$ ; Figure 1). Without normalization to GAPDH, CYP3A4 protein content was highest in LCLs with the *CYP3A4\*1G/\*1G* diplotype ( $2.37 \pm 0.23$  relative fluorescent units), intermediate for heterozygote *CYP3A4\*1/\*1G* ( $1.48 \pm 0.27$ ), and lowest for *CYP3A4\*1/\*1* ( $0.92 \pm 0.05$ ,  $p < 0.01$ ). GAPDH was not statistically different between *CYP3A4* diplotypes ( $p = 0.16$ ).

## CYP3A4 protein content and activity in human liver microsomes

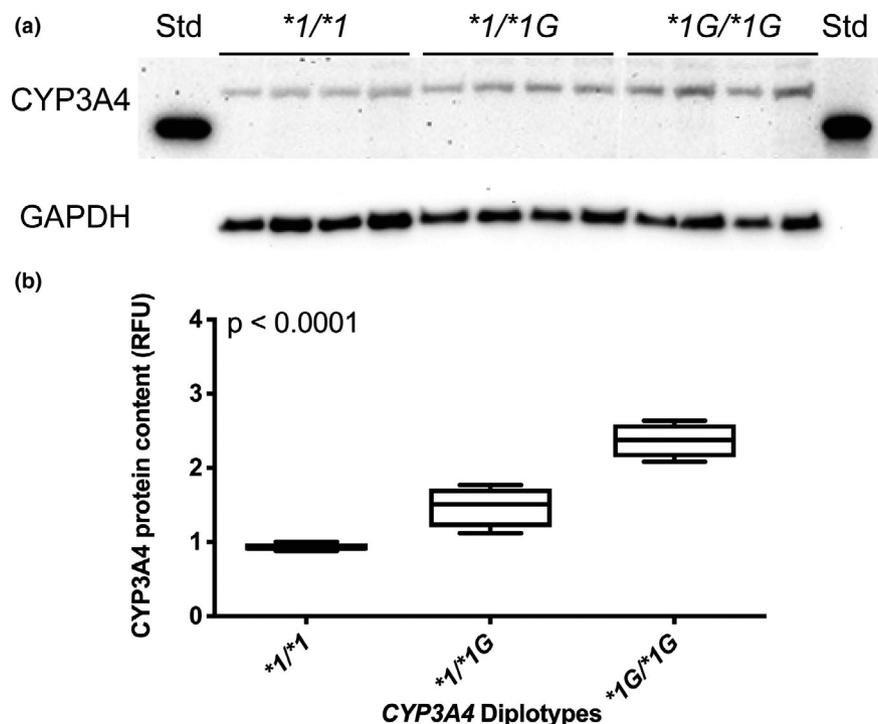
Adjusting for site of sample collection, HLM samples with a *CYP3A4\*1G/\*1G* diplotype exhibited a statistically significant higher level of log transformed CYP3A4 protein content ( $p < 0.01$ ; Figure 2A) and log transformed CYP3A4 activity measured with a CYP3A4-specific substrate ( $p = 0.04$ ; Figure 2B) than did samples homozygous or heterozygous for the reference allele at that position. CYP3A4 protein content (mean  $\pm$  SD) was  $63.1 \pm 62.9$ ,  $54.6 \pm 56.0$ , and  $221 \pm 325$  pmol/

**TABLE 2** Allele frequencies in SCF ( $n = 363$ ) and YK ( $n = 350$ ) populations compared with other populations globally

rs number	Allele name	Reference allele	Variant allele	Protein effect	SCF	YK	CSKT	AMR	AFR	EAS	EUR	SAS
Number of alleles:	Allele name	Reference allele	Variant allele	Protein effect	726	700	188	694	1322	1008	1006	978
CYP3A4 Allele frequency (%) <sup>a</sup>												
rs2242480	CYP3A4*1G	C	T	Intron 10	12.7 (10.3–15.1)	4.0 (2.5–5.5)	26.8	39.3	85.0	26.8	8.2	37.1
rs2740574	CYP3A4*1B	T	C	5' flanking	3.0 (1.8–4.2)	0	2.2	10.5	76.6	0.4	2.8	4.0
rs55785340	CYP3A4*2	T	C	S222P	0	0	0	0	0	0	0.2	0
rs4646438	CYP3A4*6	Del	A	277 frameshift	0.6 (0.0–1.2)	0	0	ND	ND	ND	ND	ND
rs56324128	CYP3A4*7	G	A	G56D	0	0	0	0	0	0	0.2	0
rs12721627	CYP3A4*16	C	G	T185S	0	0	0	0	0	0.4	0	0
rs28371759	CYP3A4*18	T	C	L293P	0	0	0	0	0	1.1	0	0.6
rs35599367	CYP3A4*22	C	T	Intron 6	2.4 (1.3–3.5)	0.3 (0.0–0.7)	2.4	2.6	0.1	0	5.0	0
CYP3A5 Allele frequency (%) <sup>a</sup>												
rs28365083	CYP3A5*2	C	A	T398 N	0.3 (0.0–0.7)	0.0	0	0	0	0	0.4	0
rs776746	CYP3A5*3	T	C	Splice	89.8 (87.6–92.0)	95.9 (94.4–97.4)	92.5	79.9	18.0	71.3	94.3	37.1
rs10264272	CYP3A5*6	C	T	Splice	0.6 (0.0–1.2)	0.0	0	2.3	15.4	0	0.3	4.0
rs41303343	CYP3A5*7	Del	A	346 frameshift	0.1 (0.0–0.3)	0.0	0	0.3	11.8	0	0	0
rs28383468		C	T	H30Y	0.4 (0.0–0.9)	0.0	0	0.1	0	0	1.2	0
rs28365085		T	C	I488 T	0.4 (0.0–0.9)	0.0	0	0	0	0.8	0	0
rs547253411		G	Del	458 frameshift	0.3 (0.0–0.7)	0.0	0	ND	ND	ND	ND	ND
rsNA		C	T	P484L	0.1 (0.0–0.3)	0.0	0	ND	ND	ND	ND	ND

Abbreviations: AFR, African, including Yoruba in Ibadan, Nigeria; Luhya in Webuye, Kenya; Americans of African Ancestry in SW USA; Gambian in Western Divisions in the Gambia; Mendé in Sierra Leone; Esan in Nigeria; AMR, Ad Mixed American, including Puerto Ricans from Puerto Rico; Colombians from Medellin, Colombia; Peruvians from Lima, Peru; Mexican Ancestry from Los Angeles, USA; CSKT, Confederated Salish Kootenai Tribes; EAS, East Asian, including Han Chinese in Beijing, China; Southern Han Chinese; Japanese in Tokyo, Japan; Chinese Dai in Xishuangbanna, China; Kinh in Ho Chi Minh City, Vietnam; EUR, European, including Utah Residents with Northern and Western European ancestry; Finnish in Finland; British in England and Scotland; Iberian population in Spain; Toscani in Italy; ND, not determined; rsNA, indicates a SNV without a reference identification number; SAS, South Asian, including Gujarati Indians from Houston Texas; Punjabi from Lahore, Pakistan; Bengali from Bangladesh; Sri Lankan Tamil from the UK; Indian Telugu from the UK; SCF, Southcentral Foundation; YK, Yukon Kuskokwim.

<sup>a</sup>Global frequencies are from the 1,000 genomes Phase 3 database, as accessed through Ensembl (<http://uswest.ensembl.org/index.html>) on July 15, 2020, and compared with the Confederated and Salish Kootenai Tribes (CSKT).<sup>34</sup>



**FIGURE 1** CYP3A4 protein content in lymphoblastoid cell lines (LCLs). Immunoblot of CYP3A4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in LCLs with *CYP3A4*\*1/\*1, \*1/\*1G, and \*1G/\*1G diplotypes (panel a). Quantitation of CYP3A4 normalized to GAPDH endogenous control (panel b). One-way analysis of variance was used to compare *CYP3A4* diplotypes

mg protein for *CYP3A4*\*1/\*1, \*1/\*1G, and \*1G/\*1G diplotypes, respectively. CYP3A4 activity (mean  $\pm$  SD) was  $0.046 \pm 0.076$ ,  $0.040 \pm 0.065$ , and  $0.116 \pm 0.148$  pmol/mg protein/min for *CYP3A4*\*1/\*1, \*1/\*1G, and \*1G/\*1G diplotypes, respectively. Log POR content ( $p < 0.01$ ), *AKR1D1* content ( $p < 0.01$ ), site of sample collection ( $p = 0.02$ ), age ( $p < 0.01$ ), sex ( $p < 0.01$ ), and *CYP3A4*\*22 genotype ( $p = 0.01$ ) each were individually significantly associated with log CYP3A4 protein content. Log POR content ( $p < 0.01$ ), *AKR1D1* content ( $p < 0.01$ ), site of sample collection ( $p < 0.01$ ), and age ( $p = 0.04$ ) each were individually significantly associated with log CYP3A4 activity, but sex ( $p = 0.14$ ) and *CYP3A4*\*22 ( $p = 0.11$ ) genotype were not.

Using a recessive inheritance model and adjusting for the factors found to be significant in individual models, the *CYP3A4*\*1G/1G was no longer significantly associated with increased CYP3A4 protein content and metabolic activity in HLMs (Table 3). As expected, CYP3A4 protein content correlated strongly with CYP3A4 protein activity ( $p < 2 \times 10^{-16}$ ). Liver collection site was a significant predictor of CYP3A4 activity, which is consistent with previous studies with CYP2A6, CYP2C19, and CYP2D6 function in these liver samples.<sup>39,40,43</sup> POR protein content and *AKR1D1* mRNA content were also significant predictors of CYP3A4 protein content and activity, consistent with previous studies from these liver samples.<sup>39,40</sup> Interestingly, the *CYP3A4*\*22 allele that has been associated with altered CYP3A4 activity elsewhere, was not a

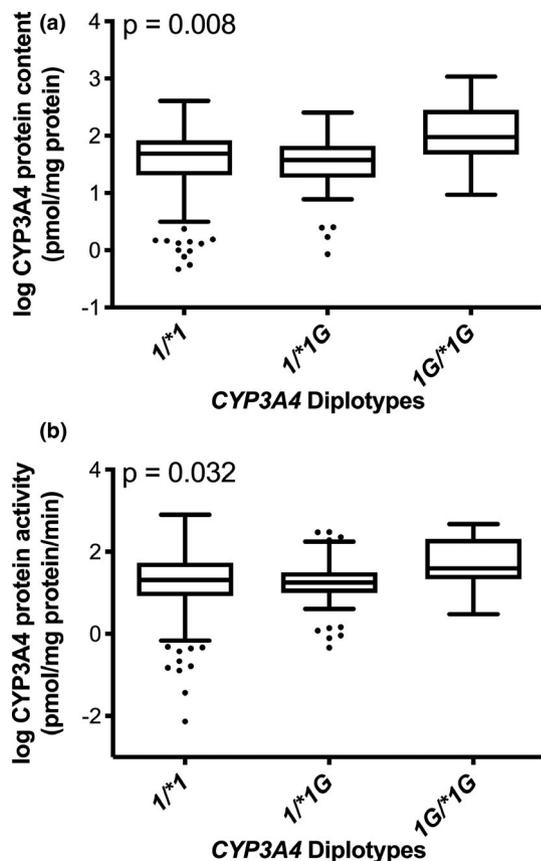
significant predictor of CYP3A4 activity, but was a significant predictor of CYP3A4 protein.

### Endogenous CYP3A4-mediated vitamin D hydroxylation

To confirm our in vitro data showing increased function of the *CYP3A4*\*1G allele, we measured the ratio of serum  $4\beta,25(\text{OH})_2\text{D}_3$  to  $25(\text{OH})\text{D}_3$  in vivo. This ratio is proportional to an individual's CYP3A4-dependent intrinsic metabolite formation clearance. Participants with at least one *CYP3A4*\*1G exhibited an increased ratio of serum  $4\beta,25(\text{OH})_2\text{D}_3$  to  $25(\text{OH})\text{D}_3$  (Figure 3), indicating that CYP3A4 activity was 17.2% higher in individuals with the *CYP3A4*\*1G allele ( $n = 57$ ) than in individuals without the *CYP3A4*\*1G allele ( $n = 457$ ,  $p < 0.01$ ). We did not have enough participants with homozygous *CYP3A4*\*1G diplotypes to analyze separately.

## DISCUSSION

Using both in vitro and in vivo data, our research demonstrates that the *CYP3A4*\*1G allele is associated with increased CYP3A4 protein content and metabolic activity compared with the reference allele. We also identified relatively low frequencies of novel and known *CYP3A4* and



**FIGURE 2** CYP3A4 protein content and activity in human liver microsomes (HLMs). CYP3A4 protein content (panel a) and CYP3A4 metabolic activity (panel b) by *CYP3A4* diplotypes: *CYP3A4*\*1/\*1 ( $n = 245$ ), \*1/\*1G ( $n = 64$ ), and \*1G/\*1G ( $n = 10$ ). All incubations were completed in triplicate. Multivariable linear regression was used to compare protein content and activity between HLMs based on genotype, adjusting for site of sample collection

*CYP3A5* alleles, including low frequencies of the functional *CYP3A5*\*1 variant, in AIAN participants from 2 regions of Alaska. Our work illustrates broad heterogeneity across American indigenous populations and suggests that AIAN people with the *CYP3A4*\*1G allele may exhibit, on average, higher metabolic activity toward CYP3A4 substrates than people from other populations worldwide.

To identify novel variants and determine the frequency of these and known variants, we resequenced *CYP3A4* and *CYP3A5* genes in AIAN participants from 2 regions of Alaska, one representing southcentral Alaska and the Aleutian Islands, and the other a region of western Alaska in the YK Delta. We identified the *CYP3A4*\*1G at lower frequencies in the SCF cohort (12.7%) and YK cohort (4.0%) than what we previously reported in the CSKT cohort (26.8%).<sup>34</sup> We also found other *CYP3A4* variants at low frequencies in both Alaskan cohorts, including the *CYP3A4*\*22 allele, which has been associated with reduced CYP3A4 mRNA and protein expression, and with poor metabolic activity toward tacrolimus and statins.<sup>5,17–19</sup> We identified very low frequencies

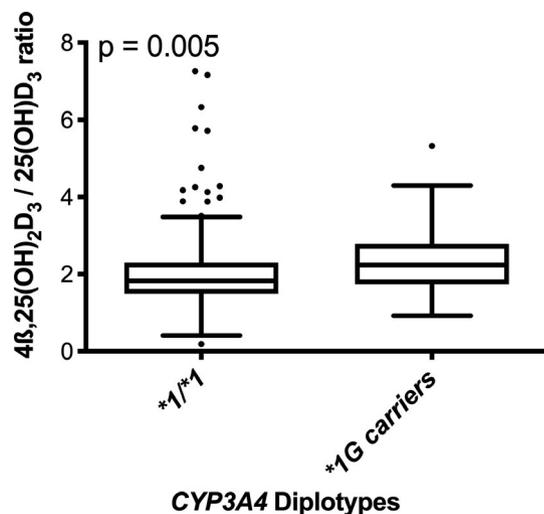
**TABLE 3** Multiple linear regression analysis of covariates with log CYP3A4 protein content and log CYP3A4 activity in human liver microsomes, adjusting for variables independently associated with log CYP3A4 protein content and activity

Covariate	$\beta$ value	Standard error	$p$ value
Log CYP3A4 protein content (pmol/mg protein)			
<i>CYP3A4</i> *1G/*1G diplotype	0.12	0.09	0.18
log POR protein content	1.06	0.13	$5.0 \times 10^{-14}$
100* <i>AKR1D1</i> mRNA content	0.30	0.97	$2.5 \times 10^{-3}$
UW sample collection	0.04	0.06	0.52
100*Age by year	0.34	0.13	0.01
Male	-0.06	0.06	0.10
<i>CYP3A4</i> *22 present	-0.21	0.09	0.03
Log CYP3A4 activity (pmol/mg protein/min)			
<i>CYP3A4</i> *1G/*1G diplotype	0.18	0.11	0.12
Log POR protein content	1.33	0.16	$1.1 \times 10^{-14}$
100* <i>AKR1D1</i> mRNA content	0.27	0.12	$2.3 \times 10^{-2}$
UW sample collection	0.30	0.08	$3.7 \times 10^{-4}$
100*Age by year	0.51	0.16	$1.9 \times 10^{-3}$

Abbreviations: POR, cytochrome P450 oxidoreductase; *AKR1D1*, aldo-keto reductase 1D1; UW, University of Washington.

of the functional *CYP3A5* allele, *CYP3A5*\*1, in both the SCF and YK cohorts, similar to our prior work with the CSKT.<sup>34</sup> These findings contrast with reported frequencies of *CYP3A5*\*1 in other North and South American indigenous populations, including ~ 60% among Pima Indians ( $n = 6$ ) and 23.7% in 2 admixed Central American indigenous populations ( $n = 232$ ).<sup>32,48</sup> Of note, the linkage disequilibrium between *CYP3A4*\*1G and *CYP3A5*\*1 was  $r^2 = 0.55$  in the SCF cohort and  $r^2 = 0.90$  in YK cohort, compared with  $r^2 = 0.158$  in the CSKT cohort.<sup>34</sup> Collectively, these differences in allele frequencies and linkage disequilibrium patterns across *CYP3A4* and *CYP3A5*, and the heterogeneity they represent, highlight the importance of introducing comprehensive genetic testing of individuals, without relying on preconceived racial and ethnic assumptions, if genetic information is to be used optimally to guide drug therapy decisions.

The *CYP3A4*\*1G allele has been associated with increased clearance of amfepramone,<sup>20</sup> tacrolimus,<sup>21,22</sup> imatinib mesylate,<sup>23</sup> and finasteride.<sup>24</sup> Additionally, the *CYP3A4*\*1G allele has been associated with lower rates of clopidogrel resistance<sup>25,26</sup> and higher rates of sodium valproate-response in pediatric patients.<sup>27</sup> Data are conflicting for vitamin D, however, with *CYP3A4*\*1G associated with higher levels of 25-hydroxyvitamin D in pregnant women in one study,<sup>28</sup> but lower levels in another.<sup>29</sup> Furthermore, clinical data emerging



**FIGURE 3** CYP3A4-mediated vitamin D hydroxylation. Ratio of serum  $4\beta,25(\text{OH})_2\text{D}_3$  and  $25(\text{OH})\text{D}_3$  in unrelated participants from the Yukon-Kuskokwim (YK) Delta ( $n = 514$ ) by *CYP3A4* diplotypes: individuals with no *CYP3A4\*1G* ( $n = 457$ ) and individuals who at least one copy of *CYP3A4\*1G* ( $n = 57$ ). A *t*-test was used to compare the two groups

primarily from eastern Asia, where the *CYP3A4\*1B* allele is not present or occurs at a very low frequency, suggest lower metabolic activity toward fentanyl<sup>11,13–15</sup> and atorvastatin<sup>7,30</sup> attributed to the *CYP3A4\*1G* allele. Our in vitro and in vivo data support the increased expression and activity of CYP3A4 with the *CYP3A4\*1G* allele. LCLs heterozygous or homozygous for *CYP3A4\*1G* had higher CYP3A4 protein content relative to LCLs homozygous for *CYP3A4\*1*. We were not able to quantitate activity in LCLs with CYP3A4 activity below the limit of detection of the assay, presumably due to relatively low levels of CYP3A4 expression. Although the CYP3A4 bands in the immunoblot from LCL lysates migrated more slowly than those from the recombinant CYP3A4 standard from baculovirus-infected insect cells, it is likely that the differences in co-migration are due to different matrices between the cell lysates and recombinant enzyme, which is consistent with the manufacturer's findings that CYP3A4 migration patterns differ by matrices.

Importantly, livers containing the *CYP3A4\*1G/\*1G* diplotype had increased CYP3A4 protein content and metabolic activity measured with a CYP3A4-specific substrate. After adjusting for covariates, including *AKR1D1* mRNA content, POR protein content, liver collection site, and age for both protein content and activity, and also sex and *CYP3A4\*22* in the case of CYP3A4 protein content, however, the association with *CYP3A4\*1G* was not significant, although still trending toward an association with increased CYP3A4 protein and activity. The association between POR protein content and CYP3A4 protein content and activity dominates any signal from the *CYP3A4\*1G* allele, which may result in a loss of power to detect the effects of *CYP3A4\*1G*. Interestingly, the

*CYP3A4\*1G* allele does not appear to be an expression quantitative trait loci in the liver according to the Genotype-Tissue Expression (GTEx) project.<sup>49</sup> Thus, whereas gene expression may not be affected, changes in protein synthesis or stability may affect protein and activity levels.

We further corroborated our in vitro results using vitamin D hydroxylation as an in vivo biomarker for CYP3A4 activity. Participants from the YK Delta with the *CYP3A4\*1G* variant exhibited increased metabolic activity as measured by the ratio of serum  $4\beta,25(\text{OH})_2\text{D}_3$  to  $25(\text{OH})\text{D}_3$ . Our group has previously shown data to validate the use of this ratio as an in vivo biomarker of CYP3A4 activity. We have shown that  $4\beta,25(\text{OH})_2\text{D}_3$  is produced from  $25(\text{OH})\text{D}_3$  predominantly by CYP3A4, with only minor (<10%) contribution from CYP3A5.<sup>50</sup> Additionally, we showed that the  $4\beta,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$  ratio is strongly correlated with midazolam hydroxylation in HLMs, and  $4\beta,25(\text{OH})_2\text{D}_3$  formation was induced by rifampin and inhibited by CYP3A4-specific inhibitors in human hepatocytes.<sup>50</sup> We also showed that  $4\beta,25(\text{OH})_2\text{D}_3$  plasma concentration increased 60% after short-term rifampin administration in healthy volunteers.<sup>51</sup> The enhanced activity we observed in individuals with the *CYP3A4\*1G* allele may extend toward other CYP3A4 substrates, such as some of the statins and tacrolimus that are of interest for genetically guided drug therapy.<sup>33</sup> The presence of the *CYP3A4\*1G* allele may necessitate increased dose requirements in individuals with this allele relative to people with the reference allele.

In summary, allele frequencies of *CYP3A4* and *CYP3A5* vary widely across subgroups of AIAN people and across the globe. Characterizing unique genetic factors that contribute to alterations in CYP3A4 and CYP3A5 function is critical for the equitable implementation of pharmacogenetic-guided therapies.<sup>52</sup> By better understanding the functional effects of these alleles, such as the *CYP3A4\*1G* allele that we demonstrate conferring increased activity, we make pharmacogenetic testing more valuable for people with understudied variants.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## AUTHOR CONTRIBUTIONS

A.E.F., K.E.T., and E.L.W. wrote the manuscript. K.E.T., and E.L.W. designed the research. A.E.F., R.D., K.S., K.J., S.E.H., R.R., B.A.K., D.A.D., and B.B.B. performed the research. A.E.F., R.D., K.S., T.A.T., and E.L.W. analyzed the data. K.G.C., B.P., E.S., and D.A.N. contributed new reagents/analytical tools.

## DISCLAIMER

As an Associate Editor of *Clinical and Translational Science*, Erica Woodahl was not involved in the review or decision process for this paper.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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