ORIGINAL ARTICLE

A pharmacogenetic study of CD4 recovery in response to HIV antiretroviral therapy in two South African population groups

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South Africa, like many other Southern African countries, has one of the highest HIV infection rates in the world and many individuals consequently receive antiretroviral therapy (ART). However, knowledge regarding (i) the prevalence of functional single nucleotide polymorphisms (SNPs) in pharmacologically relevant genes, and (ii) variance in pharmacotherapy both within and between different populations and ethnic groups is limited. The aim of this study was to determine whether selected polymorphisms in cytochrome P450 (CYP) genes (*CYP2B6* and *CYP3A4*) and the multidrug-resistance 1 (*ABCB1*) gene underlie altered antiretroviral (ARV) drug response in two South African populations. DNA samples from 182 HIV-positive individuals of Mixed-Ancestry and Xhosa ethnicity on ART were genotyped for the A-392G SNP in *CYP3A4*, the G516T and A785G SNPs in *CYP2B6*, and the T-129C, C1236T, G2677T/A and C3435T SNPs in *ABCB1*. Univariate two-way analysis of variance (ANOVA) testing revealed no apparent effect of ethnicity on immune recovery (in terms of CD4-cell count) in response to ART. Univariate one-way ANOVA testing revealed a discernible effect of genotype on immune recovery in the cases of the T-129C ($P=0.03$) and G2677A ($P<0.01$) polymorphisms in the *ABCB1* gene. This study serves as a basis for better understanding and possible prediction of pharmacogenetic risk profiles and drug response in individuals and ethnic groups in South Africa. *Journal of Human Genetics* (2009) 54, 261–265; doi:10.1038/jhg.2009.20; published online 13 March 2009

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INTRODUCTION

The scale of the HIV/AIDS epidemic and the consequent levels of need and usage of antiretroviral (ARV) drugs in South Africa necessitate clinically and cost-effective treatment programs. Despite the benefits offered by combination antiretroviral therapy (ART), it is widely recognized that all of the ARV drug classes have multiple effects other than the intended suppression of HIV replication.¹ None of the many clinical studies of ART has shown 100% response rates in terms of either control of viral replication or CD4-cell recovery.² A significant proportion of patients experience a loss of immunological, virological or clinical benefit from current regimens, whereas others suffer from adverse drug reactions (ADRs).³ Variations in ARV drug efficacy and the occurrence of related ADRs are influenced by DNA sequence variants within genes responsible for drug metabolism and transport, as well as numerous other factors, such as drug formulation, interactions, dosage and absorption. Furthermore, significant differences exist in the frequency of DNA sequence variants between ethnic groups, which can lead to marked differences in drug response between such groups.⁴ An improved understanding of the presence, frequency and possible effects of DNA sequence variants in genes

relevant to ARV metabolism and transport within South African (SA) ethnic groups could potentially aid in the customization of ART on both a population/ethnic and an individual level.⁵ In this way, the use of ineffective therapies can be minimized and valuable health resources applied more advantageously. Numerous genes involved in ARV drug metabolism and transport have been implicated in variable ART efficacy and in ARV-induced ADRs, and include members of the cytochrome P450 (CYP) gene family—most notably *CYP2B6* and *CYP3A4*—and the multidrug-resistance 1 (*ABCB1*) gene.^{6,7}

CYP2B6

Several studies^{8–14} reported an association between the *CYP2B6* G516T and A785G single nucleotide polymorphisms (SNPs) and the rate of plasma clearance of efavirenz and occurrence of related ADRs.

CYP3A4

The most-common and best-characterized SNP within *CYP3A4* is the A-392G transition in the putative nifedipine-specific element, designated as the *CYP3A4*1B* allele.¹⁵ Studies have linked this

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polymorphism to altered expression *in vitro*,¹⁶ as well as to 1.6- and 2.1-fold higher levels of nifedipine oxidation and hepatic CYP3A4 content, respectively.¹⁷

ABCB1

A number of studies have examined the possible effects of *ABCB1* sequence variants on ART response. In a landmark study, Fellay *et al.*¹⁸ detected an association of the C3435T SNP with differences in plasma drug concentrations of nelfinavir and efavirenz, in which the median drug concentration in patients with the *ABCB1* 3435 CT genotype was at the 50th percentile, compared with that of *ABCB1* 3435 TT and CC genotypes residing at the 30th and 75th percentiles, respectively ($P=0.0001$). A subsequent study by Nasi *et al.*¹⁹ failed to confirm these findings—the *ABCB1* C3435T polymorphism was found not to influence the immunological response to ART in drug-naïve HIV-positive adults. Furthermore, Winzer *et al.*²⁰ also found no significant differences in the immunological response with respect to the G2677T/A and C3435T genotypes during the first 48 weeks of ART.

An improved understanding of the genetic influences on ARV drug response could lead to improved therapies with fewer side effects and minimized drug resistance. The major aim of this study was to investigate the genetic basis for any observed differences in ART response, in terms of both drug efficacy and ADR occurrence, in the SA Xhosa and Mixed-Ancestry ethnic groups by examining selected SNPs within the *CYP2B6*, *CYP3A4* and *ABCB1* genes in patients undergoing ART.

MATERIALS AND METHODS

Study population and ART regimens

The study population consisted of SA Xhosa and Mixed-Ancestry HIV-positive patients who were enrolled for ARV treatment at the Infectious Diseases Clinic at the TC Newman Community Centre and Antenatal Clinic at Paarl Hospital in the Western Cape. The Xhosa are representative of the Nguni-speaking tribes²¹ and are originally from the eastern coast of South Africa, but have subsequently migrated to other areas within the country. The Mixed-Ancestry

(Colored) population is of Asian, Western European and Southern and Eastern African ancestry.²² This study (Project Number: N04/10/181) received ethical approval from Committee for Human Research of the Faculty of Health Sciences at Stellenbosch University.

Patients were started on first-line therapy, which comprised a combination of three simultaneously administered drugs, namely, lamivudine, stavudine and efavirenz. Patients who were pregnant received only two drugs—nevirapine and zidovudine—for prevention of mother-to-child transmission, but also qualified for triple therapy (efavirenz replaced by nevirapine) if their CD4 count was <200 cells mm^{-3} and/or they were at clinical stage IV of the disease according to the World Health Organisation (WHO) Clinical Staging System for HIV and AIDS for Adults and Adolescents. If there was no response to ART, the regimen was changed to second-line therapy, which also consisted of three simultaneously administered drugs, namely, didanosine, zidovudine and lopinavir/ritonavir.

Assessment of ART efficacy and toxicity

Six months after initiation of treatment, drug response in all patients was measured by a CD4-cell count and viral load measurement. Subsequent measurements were taken every 6 months, except in cases in which the patients experienced an increase in viral load after 6 months despite ART; in such cases, a repeat viral load measurement was usually performed after another 3 months. Side effects were clinically assessed at 2, 4, 8 and 12 weeks, and thereafter at 12-week intervals.

Genotyping of patient samples

A total of 182 whole blood samples were collected from patients. Total genomic DNA was isolated from leukocytes of whole blood samples using a modified salting-out method as described earlier by Miller *et al.*²³ polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for accurate genotyping of the selected SNPs.

PCR of relevant amplicons

Sequences of the primers that were used and amplicon product sizes are presented in Table 1.

RFLP analysis

Restriction enzymes that were previously used in other studies for the genotyping of *ABCB1* T-129C (*MspAII*;²⁴), *ABCB1* G2677T/A (*Alw2II*;²⁴)

Table 1 Primers used for PCR of relevant amplicons

Gene	SNP	Wild-type	Variant	Primer sequence (5' to 3')	Product size
<i>CYP3A4</i>	A-392G	ggcaAgaga	ggcaGgaga	F: -GGACAGCCATAGAGACAAGGGgA R: -TACTGGGGAGTCCAAGGGTTCG	107 bp
				F: GGTCTGCCATCTATAAAC R: CTGATTCTTCACATGTCTGCG Lamba <i>et al.</i> ³⁶	
<i>CYP2B6</i>	G516T	tccaGtcca	tccaTtcca	F: GTAGTCCTAACATGTCAG R: AGAGCCTACA GTGCTCCCA Jacob <i>et al.</i> ³⁷	1474 bp
				F: TCTCGAGGAATCAGCATTGAGTCAATCC R: CTAAGGAAACGAAGAGCGGCCTCTG Cavaco <i>et al.</i> ²⁴	
<i>ABCB1</i>	T-129C	cgagTagcg	cgagCagcg	F: TTTAAACCTAGTGAACAGTCAGTTCC R: ATATCCTGTCCATCAACACTGACC Hoffmeyer <i>et al.</i> ³⁸	345 bp
				F: GTACCCATCATTGCAATAGCA R: TTTAGTTTGACTCACCTTCCgAG Cavaco <i>et al.</i> ²⁴	
	G2677T/A	aggtGctgg	aggt(T/A)ctgg	F: CTACCATGCATACATCAGAAAC R: CAGATGCTTGATACAGGTAAGGG Hoffmeyer <i>et al.</i> ³⁸	581 bp

Abbreviation: PCR, polymerase chain reaction.

Lowercase letters are indicative of mismatched nucleotides for the generation of a restriction enzyme recognition site.

and *ABCB1* C3435T (*Mbol*¹;²⁵) were used. Appropriate restriction enzymes were chosen for the genotyping of *CYP3A4* A-392G (*Mbo*II), *CYP2B6* G516T (*Bse*NI), *CYP2B6* A785G (*Sty*I), *ABCB1* C1236T (*Eco*0109I) and *ABCB1* G2677A (*Alw*2II; *Rsa*I).

Analysis of variance (ANOVA) testing for significance

Univariate two-way ANOVA testing of the change in CD4-cell count after 6 months of ART was performed on the separate Mixed-Ancestry and Xhosa sub-populations of the patient cohort. Univariate one-way ANOVA testing of the change in CD4-cell count after 6 months of ART was subsequently performed on the entire patient cohort as a whole (that is, the Mixed-Ancestry and Xhosa sub-populations pooled together), because no significant difference in the change in CD4-cell count on the basis of ethnicity was observed. In all instances of ANOVA testing, a *P*-value <0.05 was deemed statistically significant.

RESULTS

Clinical data

After the completion of genotyping of all patient DNA samples, the clinical data (which were kept blind) were made available for the 182 patients. A total of 126 patients remained after exclusion of those who had not yet started on ART, those who showed poor treatment compliance or met other exclusion criteria, such as excessive alcohol consumption, those who died before the commencement of treatment or shortly thereafter (± 2 weeks), and those for whom sufficient clinical data regarding treatment response were lacking. Thus, these 126 patients were used in subsequent ANOVA testing of their *CYP3A4*, *CYP2B6* and *ABCB1* genotypes and ART response. Ethnicity was not reported in 16 patients; therefore, univariate two-way ANOVA testing on the Mixed-Ancestry and Xhosa sub-populations could be performed on 39 and 71 patients, respectively. Allele and genotype frequencies of the excluded patients did not differ significantly from those of patients used in ANOVA testing.

The response of the 126 patients to ART, in terms of CD4-cell recovery, was overwhelmingly favorable. The average change in CD4-cell count from the commencement of therapy was an increase of $\sim 341\%$ after 6 months and an increase of $\sim 613\%$ after 1 year. The median values for the change in CD4-cell count after 6 months and 1 year of ART were increases of ~ 188 and 363% , respectively. The poorest response after 6 months and 1 year of ART were decreases in CD4-cell count of ~ 22 and 17% , respectively. The most favorable responses after 6 months and 1 year of ART were increases in CD4-cell count of ~ 1891 and 3717% , respectively. Of the 126 patients, 80 were found to be good responders to ART (documented as a rise in CD4 count > 200 cells mm^{-3} and/or improvement of the clinical disease state after 6 months of treatment) and 23 were found to be poor responders, whereas the responses of the remaining 23 patients (in terms of immune recovery) could not be ascertained because of their too recent commencement of therapy.

The most commonly observed ARV-induced ADRs within these 126 patients at 2 weeks after commencement of ART were skin rash: $n=5$ (4%), peripheral neuropathy: $n=9$ (7%), nausea: $n=2$ (1.6%), fatigue: $n=2$ (1.6%), back pain: $n=1$ (0.8%), dizziness: $n=1$ (0.8%), double vision: $n=1$ (0.8%), pruritis: $n=1$ (0.8%) and convulsions $n=1$ (0.8%). The most commonly observed ARV-induced ADRs within these 126 patients at 3 months after commencement of ART were skin rash: $n=4$ (3%), peripheral neuropathy: $n=9$ (7%), nausea: $n=1$ (0.8%), fatigue: $n=1$ (0.8%), double vision: $n=1$ (0.8%), pruritis: $n=1$ (0.8%), convulsions: $n=1$ (0.8%), liver enlargement: $n=1$ (0.8%) and lipodystrophy: $n=1$ (0.8%). A total of 18 patients (14%) developed lactic acidosis as a result of mitochondrial toxicity from nucleoside reverse transcriptase inhibitor usage. Of the patients

who experienced lactic acidosis, one consequently developed cortical blindness, which necessitated the cessation of all ART, and another patient died from the disorder. A further three patients died during the course of the study: one patient died from severe tuberculosis, whereas two died of other AIDS-related causes.

SNP genotype and allele frequencies in the patient cohort

The genotype and allele frequencies within the Mixed-Ancestry and Xhosa sub-populations are presented in Table 2. All seven SNPs within both the Mixed-Ancestry and Xhosa sub-populations of the patient cohort were found to be in Hardy–Weinberg Equilibrium.

ANOVA testing for significance

Univariate two-way ANOVA testing for significance of change in CD4-cell count after 6 months of ART detected no discernible effect of patient ethnicity on immune response to ART and, therefore, one-way ANOVA testing of the change in CD4-cell count was subsequently performed on the combined patient cohort as a whole (that is, the Mixed-Ancestry and Xhosa sub-populations pooled). Univariate one-way ANOVA testing revealed no statistically significant association between the A-392G SNP in *CYP3A4* or the G516T and A785G SNPs in *CYP2B6* and changes in CD4-cell count after 6 months of ART. Furthermore, no association was detected between the C1236T and C3435T SNPs in *ABCB1*, and the level of CD4-cell recovery after 6 months of ART.

However, one-way ANOVA testing did reveal a statistically significant association between the *ABCB1* T-129C ($P=0.03$) and G2677A ($P<0.01$) polymorphisms and immune recovery after 6 months of ART. In the case of the T-129C SNP, it appeared that the homozygous derived-allele patients experienced a greater increase in CD4-cell count compared with the homozygous ancestral patients. In the case of the G2677A SNP, there were statistically significant differences in the increase in CD4-cell count between homozygous ancestral and G2677A heterozygous patients ($P<0.01$), and between G2677T heterozygous patients and G2677A heterozygous patients ($P<0.04$)—in both cases, the G2677A patients showed greater increases in CD4-cell counts. No patients homozygous for the A-variant were observed in this study.

DISCUSSION

This study represents one of the first pharmacogenetic studies in two major SA population groups, documenting the effect of functional SNPs on immune recovery in response to ART. Univariate two-way ANOVA testing for significance in the change in CD4-cell count after 6 months of ART detected no discernible effect of patient ethnicity on immune response to ART. The results of this study do not confirm those of Wegner *et al.*,²⁶ Pfister *et al.*²⁷ and Kappelhoff *et al.*,²⁸ who reported a statistically significant effect of patient ethnicity on ARV drug response. Possible explanations for these findings include a greater degree of genetic similarity between the Mixed-Ancestry and Xhosa populations, as well as the limited sample size of our cohort. Therefore, further examination of the level of immune recovery in response to ART in diverse SA population groups is warranted, as statistically significant differences in response between less genetically congruent population groups may be detected (for example, between Caucasian and Xhosa populations).

As evident in Table 2, the Mixed-Ancestry and Xhosa sub-populations differ by a relatively minor degree in terms of genotype and allele frequencies for many of the SNPs in *ABCB1*, *CYP3A4* and *CYP2B6*. Univariate one-way ANOVA testing for significance of the change in CD4-cell count after 6 months of ART detected a discernible effect of genotype only in the cases of the T-129C ($P=0.03$) and G2677A

Table 2 Genotype and allele frequencies in the Mixed-Ancestry and Xhosa sub-populations of the patient cohort

	CYP3A4		CYP2B6			
	A-392G		G516T		A785G	
	Mixed-Ancestry	Xhosa	Mixed-Ancestry	Xhosa	Mixed-Ancestry	Xhosa
Genotype:						
Homozygous wild-type	12	5	32	47	31	48
Heterozygous	36	40	34	58	35	57
Homozygous variant	22	67	4	7	4	7
Total	70	112	70	112	70	112
Genotype frequencies:						
Homozygous wild-type	17.14%	4.46%	45.71%	41.96%	44.29%	42.86%
Heterozygous	51.43%	35.71%	48.57%	51.79%	50.00%	50.89%
Homozygous variant	31.43%	59.82%	5.71%	6.25%	5.71%	6.25%
Allele frequencies:						
Wild-type	42.86%	22.32%	70.00%	67.86%	69.29%	68.30%
Variant	57.14%	77.68%	30.00%	32.14%	30.71%	31.70%

	MDR1								
	T-129C		C1236T		G2677T/A		C3435T		
	Mixed-Ancestry	Xhosa	Mixed-Ancestry	Xhosa	Mixed-Ancestry	Xhosa	Mixed-Ancestry	Xhosa	
Genotype:									
Homozygous wild-type	41	74	39	84	GG	47	105	45	91
Heterozygous	24	34	28	27	GT	19	5	21	20
Homozygous variant	5	4	3	1	TT	2	0	4	1
Heterozygous					GA	2	2		
Total	70	112	70	112		70	112	70	112
Genotype frequencies:									
Homozygous wild-type	58.57%	66.07%	55.71%	75.00%	GG	67.14%	93.75%	64.29%	81.25%
Heterozygous	34.29%	30.36%	40.00%	24.11%	GT	27.14%	4.46%	30.00%	17.86%
Homozygous variant	7.14%	3.57%	4.29%	0.89%	TT	2.86%	0.00%	5.71%	0.89%
					GA	2.86%	1.79%		
Allele frequencies:									
Wild-type	75.71%	81.25%	75.71%	87.05%	G	82.14%	96.88%	79.29%	90.18%
Variant	24.29%	18.75%	24.29%	12.95%	T	16.43%	2.23%	20.71%	9.82%
					A	1.43%	0.89%		

($P < 0.01$) polymorphisms in the *ABCB1* gene. However, we do acknowledge that population-specific associations may be masked by the current approach. In the case of the T-129C SNP, patients homozygous for the C allele experienced the greatest increase in CD4-cell count, whereas heterozygous patients showed an intermediate level of immune recovery between that of the patients homozygous for T and C alleles. The T-129C polymorphism has earlier been associated with decreased *ABCB1* expression in numerous studies.^{29,30} Although the exact mechanism underlying the association between the T-129C SNP and transcriptional activity of *ABCB1* is largely unknown,³¹ it can be hypothesized that the decreased expression of *ABCB1* and consequently decreased phosphoglycoprotein (PGP) activity lead to a lower level of intestinal drug efflux ability within individuals carrying the -129C allele. Thus, this lower level of intestinal drug efflux ability allows for greater drug absorption and bioavailability, which, in the case of ART, causes a greater level of

suppression of HIV replication, and consequently a greater increase in CD4-cell count.

In the case of the G2677A SNP in the *ABCB1* gene, there appeared to be a statistically significant and marked increase in CD4-cell count within G2677A heterozygous patients (no homozygous variant patients were detected) compared with patients with the homozygous ancestral or G2677T heterozygous genotypes. This increased level of immune response to ART may be attributed to decreased PGP activity that resulted from the G2677A polymorphism and consequent substitution of the PGP Ala residue at position 893 with Thr. The altered PGP activity that resulted from the G2677A polymorphism potentially decreases intestinal drug efflux ability, thereby increasing drug bioavailability and immune response to ART, similar to the effects of the T-129C polymorphism.^{29,30} Furthermore, a number of other studies reported an effect of the G2677A variant on PGP activity and/or drug disposition,^{32–34} albeit with conflicting results. The reasons for the

discrepancies regarding *ABCB1* polymorphisms and PGP functionality and activity are unclear; however, differential expression of PGPs and other efflux transporters in peripheral blood mononuclear cells and specific lymphocyte subpopulations (that is, CD4) might play a more important role than intestinal disposition. In addition to confirmed allelic variant associations, a plethora of other pharmacogenetic and environmental determinants influences ART outcomes.³⁵ Henceforth, high-throughput genomic analysis and new technologies should be applied to allow for the discovery of additional genetic variants and integration of genetic and clinical data that may be applied to enhance ART response in the SA setting. We are well aware that CD8+ cell count is an additional measure in AIDS-related response studies. However, limited resources prevented us from collecting any such additional data. Despite several patient-specific drawbacks, as discussed earlier, findings from this study regarding the genetic basis of immune recovery in response to ART (that is, T-129C and G2677A within *ABCB1*) represent important initial steps towards a better understanding of ART pharmacogenetics and customization of ART in two SA populations.

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