MUTATIONS IN C-C CHEMOKINE RECEPTOR TYPE FIVE (CCR5) IN SOUTH AFRICAN INDIVIDUALS

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ABSTRACT

The importance of the CCR5 receptor in HIV infection and disease progression was recognized with the discovery of the Δ32 allele. Individuals homozygous for this mutation lack functional CCR5, and are almost completely resistant to HIV infection. Heterozygous individuals display decreased cell surface CCR5 which slows disease progression. Phenotypic expression of CCR5 is

heterogeneous and its relation to genetic mutations in the CCR5 gene is not currently known for the South African population. This provided the rationale for investigating genetic variation in low CCR5 expressers in South Africa. Flow cytometry was used to measure the phenotypic distribution of CCR5 in 245 individuals by assessing both the percentage of $CD4^{+}CCR5^{+}$ T-cells and CCR5 density. Genotypic data revealed 70 single nucleotide polymorphisms (SNPs), four insertions and the Δ32 deletion within the 65 individuals selected for sequencing. The Δ32 mutation was detected only in the Caucasian group and included a single homozygous individual with an absence of CCR5 expression. A total of eight previously described open reading frame (ORF) mutations were found in this study as well as twelve novel mutations with two in the ORF. Greater genetic diversity was present in the Black South African group, with 39 mutations being exclusive to this group.

Keywords: CCR5/ HIV/ SNP/ Δ32

INTRODUCTION

Although the devastating effect of the human immunodeficiency virus (HIV) is manifested globally, the magnitude of the infection is most prominent in sub-Saharan Africa. Despite consistent attempts to develop an effective vaccine against the virus, reliance on relatively toxic antiretrovirals (ARVs) is still the best choice of treatment.

The human immunodeficiency virus gains entry into host cells via its glycoprotein 120 receptor (gp120) which binds to host cell receptor cluster differentiation four ($CD4^{+}$) on T-lymphocytes (1). In order for the virus to enter the host cell it requires membrane fusion. This occurs via the virus' glycoprotein 41 receptor (gp41) which catalyzes the fusion of the virus to a host co-

receptor, being either C-C chemokine receptor type five (CCR5) or chemokine receptor type four (CXCR4), and anchors the virus to the host membrane. The CCR5 co-receptor is crucial for HIV infection as demonstrated by the discovery of the CCR5 delta 32 (Δ32) allele (2). The Δ32 mutation contains a 32 base pair (bp) deletion in the open reading frame (ORF) of the CCR5 gene which confers resistance to HIV in homozygous individuals and retards disease progression in heterozygotes. The mutated protein is unable to insert into the host cell membrane (2) which renders the cell resistant to HIV, as the virus cannot fuse with the host cell surface and is thereby denied entry into the cell.

The importance of Δ32 was highlighted following a study conducted in 2009 by Hutter and co-workers (3). The study demonstrated that performing a stem cell transplant on an HIV positive individual, using a histocompatibility leukocyte antigen-matched (HLA) HIV negative donor homozygous for the Δ32 mutation, rendered the HIV positive individual free of viral detection 20 months post-transplantation. An update in 2010 by the group confirmed that the HIV infected patient remains free of viral replication and his immune system has been reconstituted with that of the Δ32 homozygous donor (4). The group suggests that the individual is 'cured' of HIV.

The Δ32 mutation is most common in individuals of European descent, but is absent or rare in the Black African population (5). Other mutations have however been identified in African populations, such as the C101X mutation (6), which is known to provide resistance to HIV (7). Several novel mutations have also been identified in small cohorts in South Africa (8). These include the D2V mutation with decreased binding to HIV and the R225X mutation, which like

the Δ32 mutation, results in the absence of receptor expression and no interaction of the receptor with HIV *in vitro* (9).

In South Africa, studies have been conducted to describe previously identified mutations (10) as well as novel mutations in the population (8). While studies have been performed to evaluate the *in vitro* effect of novel South African mutations on HIV (9, 11), there are currently no studies describing CCR5 expression levels in the population groups and how these levels are influenced by genetic variability in CCR5. The above studies have also focused on discovering mutations in exon 3 of the CCR5 gene that houses the ORF. In 2010, Picton and co-workers analyzed the entire 9.2kb region of CCR5 in 35 Black and Caucasian South Africans for single nucleotide polymorphisms (SNP's), indels (inserts and deletions), and haplotypes (12). In the small cohort of individuals studied, 68 SNP's, five indels and seven haplotypes were found. Research into the effects of these mutants on CCR5 expression, HIV infection, and disease progression has not been reported.

Considering that reduced CCR5 expression has been associated with delayed HIV progression, this study used a novel approach by first determining the genotypic variation within the CCR5 gene in the South African population. By targeting individuals with phenotypically low CCR5 expression using flow cytometric methods, the study aimed to discover novel mutations in the CCR5 gene which could explain the phenotype.

MATERIALS AND METHODS

The study was approved by the University of Pretoria Health Sciences Research Ethics Committee and informed consent was obtained from all individuals.

Study Population

Peripheral blood was drawn at Steve Biko Academic hospital by trained personnel. The samples were processed within six hours of collection. Flow cytometric analysis of CCR5 expression was performed on a cohort of 245 randomly selected South African individuals, of which 124 subjects were Black and 110 Caucasian. Eleven individuals formed part of the Coloured and Indian population groups. There were no specific selection criteria.

A total of 65 individuals, identified based on their low expression levels of CCR5 by flow cytometry, were genotyped (Table I). The population genotyped included 29 Black, 35 Caucasian, and one Indian-individual. Forty five of the individuals sequenced, were identified within the lower range of CCR5 density (0-3.5), 19 individuals were within the normal range of CCR5 density (3.6-6), and one individual exceeded a density of 6 arbitrary units (Figure 1).

Whole Blood cell surface staining and CCR5 phenotypic analysis

Whole Blood (50µl) was incubated with 10 µl each of CD4-FITC (Beckman Coulter, Miami, FL) and CCR5-PE (BioLegend, San Diego, CA) monoclonal antibodies for 20 minutes. Erythrocytes were lysed with VersaLyse™ lysing solution (Beckman Coulter) and flow cytometric analysis was performed on a Beckman Coulter Cytomics FC500 Flow Cytometer. The expression of CCR5 on

Table I : Flow cytometry data of 65 individuals genotyped

CCR5 MFI Range	Black African				Caucasian			
	Sample ID	**MFI**	**$CD4^+$T cell %**	**$CD4^+CCR5^+$ %**	**Sample ID**	**MFI**	**$CD4^+$T cell %**	**$CD4^+CCR5^+$ %**
0 – 3.5	F051	1.4	50.5	12.3	F092[a]	0.0	29.4	0
	F039	1.6	7.6	25.9	F054[a]	1.3	8.7	10.4
	F037	1.8	11.1	11.3	F042[a]	1.4	4.9	10.7
	F047	2.1	14.4	31	F044	1.6	9.5	14.1
	F048	2.3	15.2	17.3	F043	1.6	20.6	5.4
	F114	2.5	51.5	34.5	F050	1.7	18.9	10.7
	F158	2.7	49	26.8	F040	1.7	10.1	18.4
	F142	2.7	27	29.2	F052	1.8	4.7	35.6
	F099	2.8	11.6	9.9	F055	1.8	11.7	12.8
	F172	2.8	48.7	5.8	F046	1.8	15	14.6
	F086	2.9	28.3	55.9	F049	1.8	7.6	20.4
	F059	3.1	13.2	13.9	F056	1.8	4.9	18.6
	F135	3.1	39.6	10.6	F053	1.9	13	8.4
	F009	3.2	26	17.9	F045	1.9	12.9	18.5
	F216	3.3	47.9	7.8	F087[a]	2.4	26.5	16.8
	F084	3.3	34.6	20	T001[a]	2.5	36.2	6.9
	F173	3.3	38.8	8.6	F157[a]	2.7	35.6	6.6
	F081	3.4	45.8	26.9	F024[a]	2.8	27.2	9.7
	F184	3.4	28.9	25.6	F014	2.9	50.1	8.35
					F002[a]	2.9	39.7	11.7
					F033[a]	3.0	47	12.3
					F029	3.1	40.2	6.8
					F030	3.1	41.4	19.9
					F154[a]	3.1	37	15.8
					F104[a]	3.1	36.4	16.7
					F103	3.2	42.8	6.7
3.6 - 6	F115	3.7	8.8	13.5	F070	3.7	36.3	12.1
	F203	4.1	30.6	4.8	F197[a]	3.8	48.2	8.3

	F102	4.1	36.5	12.6	F105	4.1	53.3	9.6
	F007	5.0	46	17.3	F195	4.2	59.3	9.5
	F119	5.0	32.4	42.6	F093	4.7	39.2	23.4
	F021	5.0	22.7	32.1	F121	5.1	24.3	41.5
	F101	5.9	53.5	9.4	F062	5.3	57.4	8.9
	F068	6.0	40.2	9.5	T002	5.5	34.8	17.2
	F200	6.0	60.4	6.1				
	F228	6.0	51.4	8.1				
> 6					F010	7.2		22.8
	F032[b]	5.2		26.5				

Footnote: [a] indicates individuals found with the Δ32 mutation [b] F032 indicates the individual of Indian descent, the flow data falls within the commonly found range in the present study (3.6-6)

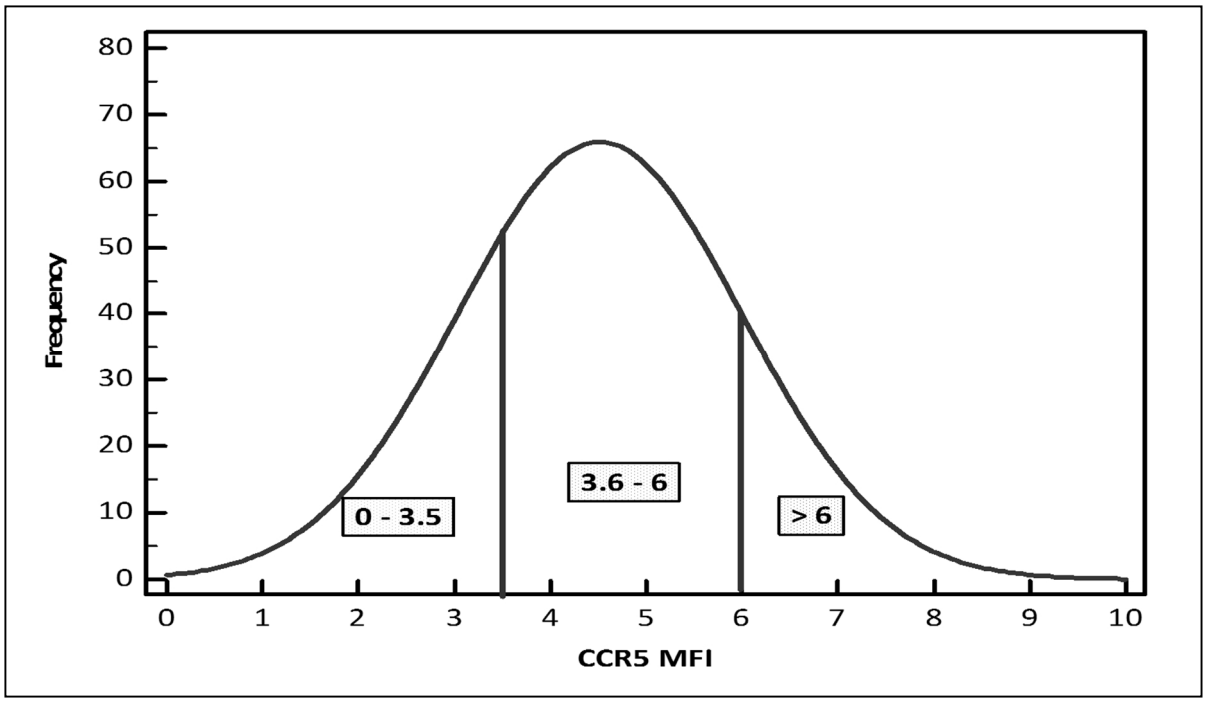

Figure 1: Distribution of CCR5 density

Histogram illustrating the distribution of CCR5 density (MFI) in 245 South African individuals as assessed by flow cytometry. MFI in most individuals was between 3.6 – 6 arbitrary units. All individuals with low CCR5 density (45) were genotyped. The remaining individuals genotyped (20) were randomly selected from the 3.6 - > 6 regions. The mean fluorescence intensity was used as a qualitative measure of density.

$CD4^+$ T-cells was analyzed after selecting the lymphocyte region using forward and side scatter and then gating for $CD4^+$ cells. The CXP system software was used to calculate the percentage of $CD4^+CCR5^+$ T-cells and the mean fluorescence intensity (MFI), which indicated qualitative CCR5 density (Figure 1), for each sample.

PCR and Sequencing of CCR5

The Maxwell® 16 Blood DNA purification kit (Promega, Madison, WI) was used to extract genomic DNA. A 9028 bp region of the CCR5 gene was amplified using four primer pairs (supplementary data) [GenBank: U95626]. The fragment amplified included the entire coding region as well as flanking regions of the CCR5 gene. Sequencing was performed utilizing an ABI version 3.1 Bigdye kit and ABI3500xl Genetic analyzer. Primers were designed using the CLC Bio Genomic workbench software program. Sequencing data was assembled and aligned with the reference sequence [GenBank: U95626] using the CLC Bio Genomic workbench software program and analyzed for the presence of SNPs, inserts, and deletions. The CCR5 gene was numbered by delineating the first nucleotide of the start of translation as +1 and the nucleotide immediately upstream as -1 (CCR5 AIDS Symposium held at the NCI-FCRDC, Frederick, MD, April 30 1999). Mutations detected in this study were compared with the GenBank dbSNP database and other published literature to identify novel mutations.

Characterization of Δ32 deletion mutant

Cloning was performed using the CloneJET™ PCR cloning kit (Fermentas Life Sciences, Inc. Maryland, USA) and for transformation Z- Competent™ E. Coli cells (Zymo Research Corporation, Irvine, CA) were used. Sequencing was used to determine the putative deletion by

screening for the allele within the recombinant plasmids. Sequencing products were aligned with the reference sequence to characterize the deletion.

RESULTS

5' Flanking region

In the 5' flanking region of the CCR5 gene 17 mutations were found which included one novel mutation (Table II). Two of the mutations detected have previously been identified in South Africa as being novel (12). The mutations in this region consisted of 15 SNPs and 2 inserts. The insert at position -3879 was found as a combination of two previously described polymorphisms with accession number rs145381188 reporting a CTAT insertion and accession number rs41412948 reporting a C/T SNP at the same location. The mutation was found in two Caucasian individuals. Seven of the mutations found are exclusive to the Black South African population while three were exclusive to the Caucasian group and included the novel C/T SNP at position -5420.

Introns

In intron 1, five SNPs were identified of which two SNPs were previously identified as being novel in the South African population (12) while one was newly identified in this study (Table II). The novel A/G SNP identified in this study was found at position -2273 in two Black South African heterozygous individuals. All 5 SNPs identified were found in the Black South African group with only the SNPs at positions -2549 and -2454 being found in the Caucasian group.

Eighteen mutations were detected in the second intron consisting of 16 SNPs and two insertions. The C/T SNP at position -1188 has been previously identified in the South African population (12) while four novel SNPs were identified in this study. These included SNPs at

position -1498, -783, -508, and -10. The C/T SNP at position -1498 was found in both the Black and Caucasian South African groups while the G/A SNP at position -783 and the C/G SNP at position -10 were found only in the Black African group. The G/A SNP at position -508 was found in a single heterozygous Caucasian individual.

Exons

A single A/G SNP was detected at position -2728 of exon 1 at a lower frequency in Black individuals than in Caucasian South Africans (Table II).

In exon 2a, four SNPs were detected at positions -2130, -2127, -2081, and -2043 (Table II). The SNPs at positions -2130 and -2081 were found at high frequencies in both the Black and Caucasian South African groups. The C/T SNP at position -2127 and the C/G SNP at position -2043 were only found in the Black South African group. There were no mutations detected in exon 2b.

ORF

Eleven mutations were detected including the Δ32 deletion and two novel SNPs (Table III). The Δ32 deletion was found in 12 Caucasian individuals and was not detected in the Black South African group. The novel C/T SNP at position 667 was found in both population groups while the A/C SNP at position 817 was only found in the Black South African group. Two mutations which have previously been identified in the South African population were also identified in this study (8).

3’ Flanking region

Nineteen SNPs were detected in the 3’ flanking region with four novel mutations being identified in this study (Table II). Five mutations have previously been identified in the South

Table II : Mutations detected in Black African and Caucasian South African individuals

Gene Region	Position	Base Change wt/m	Accession number/Reference
5' Flanking region (2715 bp)	**-5272**[b]	G/A	rs3136535
	-5270	G/A	rs6776227
	-5240	C/T	N
	-5218	T/C	12
	-5084	T/A	rs41429449
	-4749	C/T	rs3136536
	-4349[b]	A/G	rs7637813
	-4248[b]	A/C	rs41490645
	-4079	T/A	rs41499550
	-3891[b]	C/A	rs72622924/rs2856757
	-3886	T/C	rs41395049
	-3879	-/C/TTAT	rs145381188/ rs41412948
	-3875[b]	-/CTAT	rs10577983
	-3827	C/T	12
	-3453[b]	G/T	rs2734225
	-3256	G/A	rs41475349
	-2847[b]	A/G	rs2227010
Exon 1 (57 bp)	**-2728**[b]	A/G	rs2856758
Intron 1 (501 bp)	**-2572**	T/G	12
	-2549[b]	T/G	rs2734648
	-2454[b]	G/A	rs1799987
	-2449	G/A	12
	-2273	A/G	N
Exon 2a (235 bp)	**-2130**[b]	T/C	rs1799988
	-2127	C/T	rs41469351
	-2081[b]	G/A	rs1800023
	-2043	C/G	rs41355345
Intron 2 (1909 bp)	**-1830**	C/T	rs1800024
	-1681	A/C	rs9282632
	-1498	C/T	N

	-1459	A/G	rs3181037
	-1188	C/T	12
	-1126[b]	GA/-	rs3054375
	-1057	C/T	rs2856762
	-973[b]	C/T	rs2254089
	-972	G/A	rs41395249
	-783	G/A	N
	-648[b]	C/T	rs2856764
	-508	G/A	N
	-441[b]	A/G	rs2856765/rs35046662
	-359[b]	G/A	rs41515644
	-353	CAA/-	20
	-113	G/T	rs3176763
	-112	G/A	rs41352147
	-10	C/G	N
3' Flanking region (2498 bp)	**1134**	G/A	N
	1197	C/A	N
	1253	A/G	12
	1349	A/G	N
	1477	C/T	N
	1752	G/A	rs41495153
	1823	C/T	rs17765882
	1843	G/A	rs41418945
	1846	G/A	rs41466044
	2066	G/A	12
	2077[b]	T/G	rs1800874
	2225	T/C	rs41535253
	2381	A/G	12
	2435	T/A	12
	2458	A/C	rs3188094
	2676	C/A	rs41442546
	2838	C/G	rs41512547
	2919[b]	T/G	rs746492
	3132	T/G	12

[a] Accession numbers as found in the SNP database (dbSNP) or reference to article which discovered mutation. [b] Mutations found in a single Indian individual. The insertion at position -3879 is a combination of two reference sequences. For base change wt refers to wild-type allele and m refers to mutant allele. N refers to novel mutation detected in this study.

African population as being novel (12). The novel SNPs at positions +1197, +1349, and +1377 were found exclusively in the Black South African population while the SNP at position +1134 was found in a Caucasian individual.

DISCUSSION

Cell surface expression of CCR5 on $CD4^+$ T-cells is highly variable between individuals (13) and, *in vitro*, this variability has been shown to affect HIV infectability in cell lines (14), macrophages (15), and lymphocytes (16). Initially, Platt and co-workers (1998) identified a threshold of 10000 CCR5 molecules per $CD4^+$ cell as a minimal requirement for HIV infection. Levels below this threshold resulted in markedly reduced infectability but recent studies (17) have shown that specific viral isolates use lower levels of CCR5 more efficiently. Reynes and co-workers (2000) demonstrated that CCR5 expression affects virus production and viral load, and individuals with a low viral load have CCR5 densities below the threshold value (18).

The aim of this study was to analyse individuals identified by flow cytometry with a low CCR5 density for variations in the CCR5 gene. A number of mutations that are known to affect CCR5 density were found in the individuals analysed, and included the Δ32 mutation present in 11 Caucasian individuals in a heterozygous state and in a single Caucasian male in a homozygous state (Table III). The latter did not express CCR5 on the cell surface as measured by flow cytometry and is likely to be resistant to R5 HIV infection. In previous studies, the Δ32 mutation was not found in the Black African population and this was confirmed in the present study. The mutation was however found at a significantly higher frequency when compared to previous South African studies which found Δ32 at a frequency of 0.07 - 0.09 (8, 10, 12). The higher

Table III : Open reading frame mutations detected in Black Africans and Caucasians

DNA Position	Amino acid position	Base Change wt/m	Accession number[a]
105	P35	G/A	8
164	L55Q	A/T	rs1799863
180	R60S	G/T	rs1800940
303	C101X	T/A	rs1800560
554	Δ32	D32	rs333
582	Q194H	G/T	rs62625034
667	R223W	C/T	N
673	R225X	C/T	8
817	N273H	A/C	N
1004	A335V	C/T	rs1800944
1016	Y339F	A/T	rs1800945

Footnote: For base change, wt refers to wild-type allele and m refers to mutant allele. N refers to novel mutation detected in this study.

allelic frequency found in this study is possibly the result of the targeted approach in which individuals with reduced CCR5 density were sequenced. Genotypic assessment for Δ32 in individuals with higher CCR5 density will determine whether this is the case.

In the present study, the C101X mutation at nucleotide position 303 was found in a Caucasian individual who also presented with the L55Q mutation (Table III). This mutation has previously been described in the South African population in a heterozygous state and was restricted to the Caucasian group at an allelic frequency of 0.7 (10). The mutation was originally discovered in a male homozygous individual who remained uninfected despite continuous exposure from an infected partner over a 10 year period (7). Analysis of the mutant receptor showed lack of expression on the cell surface, rendering the individual resistant to HIV infection. The mutation affects an important cysteine residue involved in disulfide linkage and results in premature termination of translation with intracellular retention of the unfolded receptor (6).

A mutation exclusive to the Black and Coloured South African populations, originally discovered by Peterson and co-workers (8), was also found in the present study (Table III). The mutation at nucleotide position 673, known as R225X, was found in two Black African individuals in a heterozygous state. The mutation is found in the third ICL in which arginine is replaced by a stop codon resulting in premature termination of translation. Folefoc and co-workers (9) created a mutant receptor construct containing this mutation and found complete absence of CCR5 expression at the cell surface. Furthermore, this prevented HIV binding, and cells containing the mutant receptor were resistant to R5 HIV infection. Thus, the mutation in a homozygous state may provide resistance to HIV.

A novel SNP was detected in the intron 1 region of the CCR5 gene at position -2273 (Table II). The A/G SNP was found in two Black African heterozygous individuals. Studies have shown that the region encompassing intron 1 is crucial for CCR5 gene regulation and forms part of the Pd promoter (19, 20). The region consists of several protected areas containing important *cis* acting elements that affect CCR5 expression. The novel mutation detected in the present study affects an ATG codon. According to Mummidi and co-workers (21), these upstream ATG codons

play important roles in regulating CCR5 expression, as the upstream minicistrons that are created regulate CCR5 protein output.

A novel mutation in the ORF of the CCR5 gene was found at nucleotide position 667 and is a C/T SNP found in two heterozygous Black, and two Caucasian South African individuals (Table III). The mutation affects the third ICL of the CCR5 protein where it results in replacement of the basic arginine residue by the non-polar tryptophan, with the arginine residue being highly conserved (6). A mutation known as R223Q has previously been defined in this position and is known to significantly affect HIV co-receptor function, although it can still bind gp120 (6). The novel mutation, R223W, found in the present study may have a similar effect.

A mutation at nucleotide position 817 corresponding to position 273 in the protein was discovered in a single Black South African heterozygous individual (Table III). The individual also presented with the mutation R223W discussed above. The A/C SNP results in an amino acid change of the polar residue asparagine to the basic residue histidine (N273H). The mutation is located in the third ECL and its effect requires further investigation.

The 5'UTR, introns and 3'UTR are important in CCR5 gene regulation (21). Therefore, novel mutations found within intron 2 (Table II) may play a significant role in CCR5 expression. In particular, the C/G SNP (-10) found in a single Black South African heterozygous individual may be of considerable importance, as this region plays a significant role in translation initiation.

In conclusion, sequence analysis detected a total of 75 mutations in the study cohort. This included 63 previously known and 12 newly identified mutations. The mutations comprised 70 SNPs, four insertions, and the Δ32 mutation. Thirty nine mutations were exclusive to the Black South African group, while 13 were exclusive to the Caucasian South African group. There were no mutations detected in the 54 bp region of exon 2b. The regions with the highest number of

mutations were the 3' flanking region and intron 2. Twelve mutations thus far only identified in the South African population (8, 12) were identified in the present study.

The ORF contained nine previously identified mutations. Five of these were found exclusively in Black South African individuals while four were found in the Caucasian group only. There were no mutations in the ORF that were present in both population groups. The Δ32 mutation was found in 12 Caucasian individuals but was not detected in the Black South African group. With the exception of a single homozygous male, all individuals who displayed the Δ32 mutation were heterozygotes.

Twelve novel SNPs were identified in the present study including two in the ORF. Seven mutations were exclusive to the Black South African group while three were exclusive to the Caucasian group. Additional work is needed to determine the effect of these novel mutations on CCR5 receptor function, including ligand binding and HIV co-receptor activity. Although flow cytometry gives some indication of CCR5 surface expression, expression may not be the consequence of a single mutation. Consequently it is difficult to correlate mutations detected in the present study with level of CCR5 expression at the cell surface. However, our targeted approach, in which the majority of individuals sequenced were low CCR5 expressers, has allowed us to identify novel mutations in the CCR5 gene. As expected, greater genetic diversity was present in Black South Africans, with 39 of the mutations being exclusive to this group.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest relevant to this article.

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