Risk for HIV-1 Infection Associated With a Common *CXCL12* (*SDF1*) Polymorphism and *CXCR4* Variation in an African Population

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Summary

CXC chemokine ligand 12 (CXCL12), or stromal cell–derived factor 1 (SDF1), is the only known natural ligand for the HIV-1 coreceptor, CXC chemokine receptor 4 (CXCR4). A single nucleotide polymorphism (SNP) in the *CXCL12* gene (*SDF1*-3′A) has been associated with disease progression to AIDS in some studies, but not others. Mutations in the *CXCR4* gene are generally rare and have not been implicated in HIV-1/AIDS pathogenesis. This study analyzed the *SDF1*-3′A SNP and performed mutation screening for polymorphic markers in the *CXCR4* gene to determine the presence or absence of significant associations with susceptibility to HIV-1 infection. The study consisted of 257 HIV-1–seropositive patients and 113 HIV-1–seronegative controls representing a sub-Saharan African population belonging to the Xhosa ethnic group of South Africa. The *SDF1*-3′A SNP was associated with an increased risk for HIV-1 infection ($P = 0.0319$) whereas no significant association was observed between the occurrence of the *SDF1*-3′A SNP and increased or decreased plasma levels of CXCL12. Comprehensive mutation analysis of the *CXCR4* gene confirmed a high degree of genetic conservation within the coding region of this ancient population.

Keywords: CXC chemokine ligand 12 (CXCL12), CXC chemokine receptor 4 (CXCR4), *SDF1*-3′A single-nucleotide polymorphism, HIV-1 infection risk, African population

The role of specific chemokines acting as inhibitors of HIV-1 infection and also possibly influencing viral replication[1] was highlighted by the discovery that chemokines are natural ligands for chemokine receptors. These chemokine receptors, together with the CD4 molecule, serve as necessary cofactors for HIV-1 entry.[2–5] The CXC chemokine ligand 12 (CXCL12), known more commonly as stromal cell–derived factor 1 (SDF1), inhibits infection of T cell line–tropic (T-tropic) or syncytium-inducing viruses normally found during late-stage HIV disease[6,7]

by downregulating the surface expression of the HIV-1 coreceptor, CXC chemokine receptor 4 (CXCR4).[8,9] The demonstration that mice deficient for either CXCL12 or CXCR4 die perinatally[10] further promoted the understanding of this ligand–receptor interaction, which appears to be vital in physiological processes.

It has been found that the *CXCL12* gene, previously known as *SDF1* or *PBSF* (MIM# 600835), is located at band q11 on chromosome 10 and encodes for 2 isoforms, CXCL12α and CXCL12β, which are the result of alternative splicing of a single gene.[11–13] The coding regions for *CXCL12*α and *CXCL12*β are composed of 3 and 4 exons, respectively. The *CXCL12*β gene transcript has an extra exon that encodes for 4 additional amino acids.[13] The *CXCR4* gene, also known as *NPY3R*, *FUSIN*, and *LESTR* (MIM# 162643), is located at band q21 on chromosome 2[14,15] and consists of an intron separating 2 exons in which lies the open reading frame.[16,17]

A single-nucleotide polymorphism (SNP), designated *SDF1*-3′UTR-801G > A and abbreviated *SDF1*-3′A (rs1801157), was identified in the 3′ untranslated region (3′UTR) of the *CXCL12*β gene transcript and involves a G-to-A transition at nucleotide position +801 relative to the start codon.[18] Although the SNP in the recessive state was initially associated with delayed onset of AIDS,[18,19] other studies suggested an association with accelerated progression to death[20,21]; prolonged[21] or decreased[22] survival after AIDS diagnosis; or no effect on disease progression.[23–25] An African study showed an association between the SNP in the heterozygous state and increased vertical transmission from mother to child,[26] whereas an association with rapid disease progression and the SNP occurring heterozygously was observed in HIV-1–infected children born to seropositive mothers.[27] Recently, the SNP has been found to play a role in resistance to HIV-1 infection in seronegative high-risk individuals,[28] although this association was absent in a study involving repeatedly exposed HIV-1–seronegative subjects.[29] Studies investigating plasma CXCL12 protein levels in HIV-1–seropositive patients, exposed high-risk HIV-1–seronegative individuals, and healthy HIV-1–seronegative controls[28,30–34] with consideration of *SDF1*-3′A genotypes[32,33] have also reported inconsistent associations.

Previous studies investigating the role of *CXCR4* in host susceptibility to HIV-1/AIDS in white and African American populations have shown a relatively low occurrence of *CXCR4* mutations, and therefore their significance is unclear.[35–37] The *CXCR4* genetic variants reported include silent mutations, *CXCR4*-I261I[35] and *CXCR4*-K68K, and a nonconservative mutation, *CXCR4*-F93S.[36] Both the *CXCR4*-K68K and *CXCR4*-F93S mutations were further considered for their possible influence on HIV-1 entry, with the results being comparable to what was found for wild-type CXCR4.[36] Recently, mutations in the cytoplasmic tail domain of *CXCR4* were identified as being causative for WHIM syndrome, an immunodeficiency disorder characterized by warts, hypogammaglobulinemia, infections, and myelokathexis.[38] These mutations, however, are familial and rare.

Controversy with regards to the role of *CXCL12 (SDF1*-3′A SNP) and *CXCR4* mutations in HIV-1/AIDS pathogenesis has accentuated the need for additional studies within ethnically distinct populations. In this study we genotyped the *SDF1*-3′A SNP and performed comprehensive mutational analysis of the *CXCR4* coding region. Plasma CXCL12 levels were measured to assess possible functional correlation between the *SDF1*-3′A SNP and protein levels. Our results

indicate the importance of investigating the genetic basis for HIV-1/AIDS within specific ethnic groups, particularly populations from understudied, pandemic-stricken sub-Saharan Africa.

MATERIALS AND METHODS

Study Population

The population group represented in this study is sub-Saharan Africans, defined as individuals of Xhosa descent all residing in the Western Cape Province of South Africa. According to recent consensus, the South African population is 79% African, with the Xhosa ethnic group forming approximately 22% of the total African population and 90% of the African population residing in the Western Cape (Statistics South Africa, 2001; www.statssa.gov.za, accessed August 28, 2005). The Xhosa are from the early clan of the Nguni, the most southern group of Bantu migrants from central Africa. The HIV-1–seropositive individuals were patients of Tygerberg Hospital, Woodstock Chapel Street Community Health Clinic, or the Langa Clinic, which at the time of DNA extraction totaled 1035 ethnically diverse patients, as previously described.[39] Individuals were included in this study if they were from Xhosa descent and blood was available for DNA extraction. The HIV-1–seronegative controls were population-matched blood donors from the Western Province Blood Transfusion Service. The only criteria for exclusion was a positive HIV-1 status. Disease progression for most of the HIV-positive patients remains unknown and was therefore not assessed in this study. Informed consent was obtained from all the study participants and the Ethics Review Committee of the University of Stellenbosch approved the study protocol (#98/158). The sample size for genotyping the *SDF1*-3′A SNP consisted of 257 HIV-positive (66% female, 34% male) and 113 HIV-negative (62% female, 38% male) individuals. CXCL12 protein levels were determined for samples when plasma was available (131 HIV positive, 63 HIV negative). Comprehensive mutational analysis of the entire coding region of *CXCR4* gene was performed on 57 HIV-positive and 39 HIV-negative Xhosas. An additional 30 HIV-positive and 22 HIV-negative samples were further screened to determine allele frequencies of identified mutations.

Genetic Analysis

Genotyping

Genomic DNA was extracted from whole blood and genotyped in a blinded manner. Two methods were used to genotype the *SDF1*-3′A SNP due to availability of technologies at the time. To confirm genotyping specificity of the 2 methods, 155 samples were genotyped using both methods. The first utilized denaturing gradient gel electrophoresis (DGGE).[40] A single DGGE primer set, including a GC-clamp (GC-rich fragment) on the 5′ end of the reverse primer, was designed for partial analysis of the 3′UTR of the *CXCL12*β gene transcript: 5′-GTGAAGGCTTCTCTCTGTGG-3′ and 5′-[40GC]GTGGACACACATGATGATGG-3′. Amplification and heteroduplexing were performed as described below (mutation detection) using an annealing temperature of 56°C. Amplified polymerase chain reaction (PCR) products were electrophoresed in a 9% polyacrylamide gel with a denaturing gradient of 45%–85% urea and formamide (100% UF = 7 M urea per 40% deionized formamide), at 60°C for 110 V overnight, using the Ingeny phorU-2 system (Ingeny, Goes, The Netherlands; www.ingeny.com).

The 5′ nuclease or TaqMan allelic discrimination method[41] was also used for genotyping the *SDF1*-3′A SNP using the following primers: 5′-CAAAGCCTAGTGAAGGCTTCTCTC-3′ and 5′-TCAGGGTAGCCCTGCTGC3′; and probes: 5′-FAM-TGGGAGCC**G**GGTCTGCCTCT-TAMRA-3′ and 5′-VIC-ACATGGAGCC**A**GGTCTGCCTCTT-TAMRA-3′. PCR reactions each containing 5 ng of DNA and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) were used for amplification (detailed PCR reaction mix protocol available on request) with the cycling conditions including a initial denaturation of 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 15 seconds and annealing at 58°C for 1 minute. Allele discrimination and genotype determination were based on the endpoint fluorescence measured by the ABI7900 high-throughput sequence detection system (Applied Biosystems). A total of 199 and 326 samples were genotyped using DGGE and TaqMan allelic discrimination, respectively.

Mutation Detection

DGGE primers were designed for the entire coding region, including the intron/exon boundaries of the *CXCR4* gene. The *CXCR4* coding region within exon 1 (codons 1–5) and exon 2 (codons 6–352) was divided into 7 overlapping amplicons (A–G) (Table 1). Each PCR reaction contained 50 ng of DNA (detailed PCR reaction mixture protocol available on request) and amplification was performed using a 9600 thermocycyler (Applied Biosystems). PCR cycling conditions included an initial denaturation at 96°C for 3 minutes, followed by 32 cycles of denaturation at 96°C for 45 seconds, annealing for 1 minute (annealing temperatures are shown in Table 1), and elongation at 72°C for 1 minute, 20 seconds. Following the last cycle was an additional extension step of 72°C for 7 minutes. For heteroduplex formation, the PCR products were subjected to denaturation at 96°C for 10 minutes, followed by renaturation for 45 minutes at 56°C. Electrophoresis was used to check the amplified products, where 10% of each sample was resolved on 2% agarose gel. Optimal DGGE analysis was achieved using previously described conditions for broad-range mutation detection by DGGE.[42] Amplified PCR products were electrophoresed in a 9% polyacrylamide gel with a denaturing gradient of 30%–75% UF, at 60°C for 110 V overnight. The 7 amplicons for *CXCR4* were electrophoresed in 5 lanes (fragments B and E; fragments C and G were pooled) and allowed for the complete analysis of 6 patients per denaturing gel. The gels were stained with ethidium bromide and photographed under an ultraviolet transilluminator. Samples showing aberrant DGGE banding patterns were purified using the high pure PCR product purification kit from Roche Diagnostics (Mannheim, Germany) and subjected to automated sequencing using the non-GC-clamped primer and the dye terminator sequencing kit (Applied Biosystems; www.appliedbiosystems.com).

Determining Plasma CXCL12 Protein Levels by ELISA

Plasma was isolated by centrifugation of ethylenediamine tetra-acetic acid–anticoagulated blood samples at 2000 rpm for 10 minutes. Plasma samples were stored at −80°C before being thawed for analysis. A CXCL12 enzyme-linked immunosorbent assay (ELISA) was developed using commercial monoclonal antibodies (R&D systems, Minneapolis, MN) according to manufacturer's recommendations. Flat-bottom 96-well microtiter plates with high-binding capacity (Nunc Maxisorp, Nunc, Denmark) were coated with capture antibody (mouse antihuman CXCL12) prior to the addition of plasma samples. Biotinylated mouse antihuman

TABLE 1

CXCR4 Primer Sets and Experimental Conditions for PCR Amplification and DGGE

				Temperature (°C)	
Fragment	**Forward Amplimer, 5′-3′**	**Reverse Amplimer, 5′-3′**	**Size (bp)**	**Melting**	**Annealing**
A	CTCCAGTAGCCACCGCATCT	[40GC]GCTGCGCTCTAAGTTCAAACG	154	73	62
B	[40GC]GAATGTCCATTCCTTTGCCTCT	GCCTGTACTTGTCCGTCATGC	286	73	60
C	[40GC]CCACCATCTACTCCATCATC	AGACGCCAACATAGACCAC	397	66	55
D	CACGCCACCAACAGTCAGA	[40GC]AGCAGGACAGGATGACAATACC	278	71	60
E	[40GC]CAGTTTCAGCACATCATGGT	AGGATGAGGATGACTGTGGT	180	66	55
F	CATCTCCAAGCTGTCACACT	[40GC]TTACATCTGTGTTAGCTGGAGT	445	66	54
G	TCCACTGAGTCTGAGTCTTCAA	[40GC]TCCTGCCTAGACACACATCA	282	67	54

GC-clamp used was as follows: [40GC] CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG.

CXCL12β was used as detection antibody. Recombinant human CXCL12β was included as a standard. Each sample was run in duplicate and the mean concentration (pg/mL) of plasma CXCL12 protein was determined from the standard curve using ELISA software (Bio-Tek KC4, Bio-Tek Instruments, Winooski, VT).

Statistical Analysis

The allele and genotype distributions, including Hardy–Weinberg equilibrium estimations, were calculated. Testing of HIV-1–seropositive vs. HIV-1–seronegative subjects for significance of heterogeneity in allele and genotype frequencies was based on the 2-sided Fisher exact test for 2 × 2 contingency tables and the χ^2 test for independence, respectively. The Mann–Whitney *U* test was used to compare mean plasma CXCL12 protein levels between the case and control groups (GraphPad Software, Inc., San Diego, CA, and SAS Institute, Inc., Cary, NC).

RESULTS

Analysis of the *SDF1*-3′A SNP

TABLE 2

Allele and Genotype Distribution of the *SDF1*-3′G > A SNP in 257 HIV-1–Infected Cases vs. 113 HIV-1–Seronegative Controls From the Xhosa Population

SNP	HIV+ (%) (n = 257)	HIV− (%) (n = 113)	*P*value*
SDF1-3′G > A			
G	495 (96.3)	224 (99.1)	**0.0319**
A	19 (3.7)	2 (0.9)	
GG	239 (93)	111 (98.2)	0.1191
GA	17 (6.6)	2 (1.8)	
AA	1 (0.4)	0	
	P_{HWE} = 0.26	P_{HWE} = 0.92	

*Two-sided Fisher's exact or χ^2. $P < 0.05$ was required for statistical significance and is presented in bold.

Number of alleles, HIV+ (2n = 514) and HIV− (2n = 226).

HIV+ indicates HIV-1 seropositive; HIV−, HIV-1 seronegative; HWE, Hardy–Weinberg equilibrium.

The commonly reported *SDF1*-3′A SNP was detected using gel-based DGGE and a TaqMan allelic discrimination assay (Table 2). The 100% concordance observed for 155 samples screened with both methods reflects the reliability of the 2 *SDF1*-3′A SNP assays for generating valid and reproducible results. The presence of the SNP in the Xhosa population was observed at a significantly higher allelic frequency in the HIV-1–seropositive patients (0.037; 19/514) compared with their uninfected counterparts (0.009; 2/226), with a *P* value of 0.0319. No significance was found for the independent genotype analysis ($P = 0.1191$); however, a significant association between the presence of the A allele (AA and GA) and HIV-1 infection was observed ($P = 0.0454$). There was no significant deviation from the expected Hardy–Weinberg equilibrium in either the cases or controls. There were no significant associations between specific CXCL12 plasma levels between *SDF1*-3′A genotype among cases and controls.

Analysis of *CXCR4* and Identification of Mutations

We identified 1 previously known and 3 novel mutations in the *CXCR4* gene. The previously found silent mutation occurs in the coding region at codon 138 (rs2228014). The *CXCR4*-I138I mutation was detected using the amplicon C primers set and was observed in 1 HIV-1–seropositive patient and 1 HIV-1–seronegative control. The 3 novel mutations found in the 3′UTR at nucleotide positions +29 (G > A), +34 (A > T), and +46 (deletion T), relative to the stop codon, were all identified using the primer set for amplicon G. Further screening in additional samples resulted in the novel mutations occurring at allele frequencies ranging from 0.008–0.011 in the Xhosa population. No associations were found with susceptibility to HIV-1 infection or disease progression to AIDS.

DISCUSSION

Our study focused on the analysis of the *SDF1*-3′A SNP and the *CXCR4* gene within the Xhosa ethnic group from South Africa. The importance of determining population-specific genetic variants influencing HIV-1 susceptibility in the understudied African populations is evident.[43] A previous study by Ramaley et al[44] suggested that caution should be used when considering an association observed in one population to be present in another and showed that the *CCR5* alleles previously identified and significantly associated with influencing susceptibility to HIV-1/AIDS in white populations did not have the same effect in Africans. We have previously screened for the well-documented *CCR5*-Δ32 HIV-1/AIDS resistance-associated mutation and found it to be completely absent in the Xhosa population.[45] Although genetic markers may vary in frequency across populations, a recent study has suggested that their biologic impact on the risk for the disease may usually be consistent across traditional ‘‘racial’’ boundaries.[46] In the ancient Xhosa population, these functional genetic markers may, as yet, not have been identified in the majority of studies that have focused on younger populations.

Although the allele frequency observed for the *SDF1*-3′A SNP in our Xhosa group (0.028) is within the range previously reported for African populations,[18,20,47,48] it is slightly higher than that reported in another South African study (0.010).[47] The study participants reported by

Williamson et al[47] resided in the Free State Province of South Africa and are predominantly of Sotho ethnic descent, which could explain this bias in allele frequencies.

The *SDF1*-3′A SNP analysis resulted in an association being observed between the presence of the A allele and an increased rate of infection both at the level of allele frequency ($P = 0.0319$) and A allele carriage frequency ($P = 0.0454$), although significance was not found for independent genotype analysis. Previous studies focusing on predominantly white populations have not reported similar findings but rather found associations with disease progression to AIDS. An association with the *SDF1*-3′A SNP occurring heterozygously and increased vertical transmission from mother to child was, however, previously reported in Africans.[26] Controversies in genetic association studies have been significantly addressed in the literature, with the most compelling shortcoming of this study being the relatively small study numbers, which could result in possible bias. Therefore, these findings require independent elucidation.

Plasma CXCL12 protein levels were not significantly different between the wild-type and the *SDF1*-3′A SNP within cases and controls. Due to the relatively small sample number (as well as lack of homozygous individuals) in the study population, we determined the CXCL12 protein levels in an additional 48 HIV-seronegative (27 wild-type, 15 heterozygous, and 6 AA homozygous) white samples (data not shown) but also found no significant association. Our findings require further investigation with larger sample numbers and replication in other cohorts supporting the effect of the SNP in ligand expression. This may, however, be limited by the low occurrence of homozygotes in the Xhosa population. An advantage of our study was the use of the recombinant human CXCL12β for the ELISA assay as opposed to recombinant human CXCL12α used in other studies in which associations were reported. The latter studies included low plasma CXCL12 levels found in uninfected persons homozygous for the *SDF1*-3′A SNP[33] and a significant increase in CXCL12 levels being observed in the HIV-1–seropositive individuals when compared with the HIV-1–seronegative control group.[34]

The identification of 3 novel genetic variants within the 3′UTR of *CXCR4*, a potential regulatory region, could have an effect on the expression or functioning of the protein. CXCR4 serves as the coreceptor for T-tropic or syncytium-inducing viruses normally emerging during late-stage HIV disease,[5] and thus a possible effect of mutations occurring in *CXCR4* is more likely to be seen if they influence disease progression rather than susceptibility to HIV-1 infection. We screened an additional 51 white control subjects (data not shown), which excluded the presence of the 3 novel *CXCR4* mutations. Therefore, although no associations could be made, we can conclude that these novel variants are exclusive to the African population.

Controversy exists in the literature between the association of the *SDF1*-3′A SNP and HIV-1 infection or disease progression to AIDS. It was recently reported that other polymorphisms in linkage disequilibrium with the *SDF1*-3′A SNP, rather than the SNP itself, are responsible for altered levels of *SDF1* transcripts. The inconsistent findings for the *SDF1*-3′A SNP among various populations may therefore be attributed to different haplotype structures, including or excluding functional variants, for specific ethnic groups.[49] In this study, we found an association between the presence of the *SDF1*-3′A SNP and risk for HIV-1 infection in a sub-Saharan African population. Our results emphasize the need for investigating HIV-1/AIDS candidate genes in many diverse ethnic groups, particularly in the populations most affected by the HIV-

1/AIDS pandemic. Although this study focused on a relatively small number of individuals, its findings contribute to the growing evidence that the presence and effects of genetic variants in the understudied African populations are important when predicting host susceptibility to HIV-1/AIDS within sub-Saharan Africa.

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