HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS

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Objective: To assess syncytium-inducing (SI) and non-syncytium-inducing (NSI) frequencies, coreceptor usage and *gp120 V3* sequences of HIV-1 isolates from Ethiopian AIDS patients.

Patients: Cross-sectional study on 48 hospitalized AIDS patients (CD4 T cells $< 200 \times 10^6$ cell/l) with stage III or IV of the WHO staging system for HIV-1 infection and disease.

Methods: Peripheral blood mononuclear cells (PBMC) from all 48 patients were tested by MT-2 assay to determine SI/NSI phenotypes. Lymphocyte subsets were enumerated using Coulter counting and FACScan analysis. Viral load determination used a nucleic acid sequence-based amplification assay (NASBA). Coreceptor usage of HIV-1 biological clones was measured using U87 CD4/chemokine receptor transfectants and phytohemagglutinin-stimulated PBMC of healthy donors with wild-type *CCR5* and homozygous mutation *CCR5*Δ32 (a 32 base-pair deletion in *CCR5*). Reverse transcriptase polymerase chain reaction sequencing was performed on the third variable region (*V3*) of the HIV-1 gene *gp120*. Sequence alignments were done manually; phylogenetic analyses used PHYLIP software packages.

Results: SI viruses were detected for 3/48 (6%) AIDS patients only. Lower mean absolute CD4 counts were determined in patients with SI virus compared with NSI ($P = 0.04$), but no differences in viral load were observed. All patients were found to be infected with HIV-1 subtype C, based on *V3* sequencing. NSI biological clones used CCR5 as coreceptor; SI biological clones used CXCR4 and/or CCR5 and/or CCR3.

Conclusions: Ethiopian patients with HIV-1 C-subtype AIDS harbour a remarkably low frequency of SI phenotype viruses. Coreceptor usage of these viruses correlates with their biological phenotypes.

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Keywords: HIV-1, C-subtype, AIDS, SI, NSI, coreceptor, Ethiopia

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Introduction

HIV-1-infected individuals harbour a swarm of closely related viruses. These HIV-1 variants have been shown to differ in biological properties such as replication rate, cell tropism and syncytium-inducing capacity (SI) [1–4]. Viruses isolated in the early asymptomatic phase of infection are predominantly slow-replicating, macrophage tropic and do not have a syncytium-inducing capacity (NSI) *in vitro* [3,5]. During progression to disease, rapidly replicating T cell tropic viruses appear in about 50–70% of individuals infected with various HIV-1 subtypes. These viruses are associated with the appearance of SI HIV-1 variants [6–8]. Emergence of these SI HIV-1 variants is a predictor for accelerated progression towards AIDS [3,6–10].

The genomic diversity underlying the above phenotypic diversity of HIV-1 has been mainly assigned to the variable regions of the gene *gp120* for the envelope protein gp120 [11–13]. Sequence analysis of the V3 regions of HIV-1 SI isolates from subtype B viruses has indicated that positively charged amino acids at positions 11 and 25 are associated with the SI phenotype [12,14]. Several studies have confirmed this in subtype B and in subtypes A, D, E and O [8,14–16]. However, for HIV-1 subtypes C, F, G, H, I and J this association has not been confirmed to date.

The interest in SI/NSI phenotypes revived when it was shown that macrophage-tropic NSI HIV-1 strains use primarily the β-chemokine coreceptor CCR5 and T cell-tropic SI strains the α-chemokine coreceptor CXCR4 as coreceptors for infection of CD4 cells [17–19]. Additional β-chemokine receptors, such as CCR3, CCR2b and STRL33, have also been shown to function as coreceptors for some, but not all, primary strains of HIV-1 [17–20].

The first Ethiopian HIV-1 positive sera were detected in 1984, the first Ethiopian AIDS case was reported in 1986 and the epidemic is a rapidly growing problem in Addis Ababa, the capital city with more than 2 million inhabitants [21,22]. Earlier studies have indicated the predominant presence of HIV-1 C subtype [23–25] in Addis Ababa, but the distribution of NSI versus SI viruses in the Ethiopian HIV-1 epidemic is not documented. More information on biological characteristics of subtype C strains is of importance since, according to UNAIDS, 48% of HIV-1-positive individuals worldwide (equivalent to an estimated 14 680 000 subjects) were infected with HIV-1 subtype C by the end of 1997 [26]. This study assesses SI/NSI frequencies, coreceptor usage and underlying genetic features of *gp120 V3* of HIV-1 isolates from Ethiopian AIDS patients.

Materials and methods

Study subjects and sample collection

Forty-eight hospitalized Ethiopian patients with AIDS (CD4 T cells $< 200 \times 10^6$ cells/l) and clinical stage III or IV of the WHO staging system for HIV-1 infection and disease [27] were studied. Venous blood was drawn in EDTA vacutainer tubes (Becton & Dickinson), plasma was separated by centrifugation and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density centrifugation for 20 min at room temperature and 2200 r.p.m.

Enumeration of lymphocyte subsets

Lymphocyte subsets were enumerated in whole blood using Simultest monoclonal antibodies (mAbs) combinations CD4/CD45, CD3/CD19, CD3/CD4, CD3/CD8 and CD3/CD16+56 and isotype controls with Simulset software on a FACSCAN flow cytometer (Becton Dickinson, USA), according to the manufacturer's recommendations. In brief, 100 µl EDTA-treated blood was incubated with each combination of mAb for 15 to 20 min at room temperature in the dark. Red blood cells were lysed by adding 2 ml lysing solution per tube (FACSlyse, Becton Dickinson) and incubating for 10 min at room temperature in the dark. The cells were centrifuged at $300 \times g$ for 5 min and then washed twice with Isoton (Becton Dickinson). Stained cells were analysed by FACScan using Simulset software on a minimum of 2500 acquired events, according to CDC criteria of quality control for enumeration of lymphocyte subsets [28].

Viral load determination

Plasma viral load was measured using a nucleic acid-based amplification assay (NASBA, Organon Teknika, the Netherlands) on 100 µl samples, as described [29]. In brief, RNA was extracted from plasma using an established guanidinium isothiocyanate-based method [30] and isothermally amplified in the presence of three RNA standards using NASBA kits (Organon Teknika). Detection of amplified RNA was performed on a NucliSens reader (Organon Teknika), using the RNA standards for calibration and calculation of viral loads. Results were presented as $\log_{10}$ RNA copies/ml plasma.

Assay for MT-2 and p24 ELISA

For the MT-2 assay, 1×10^6 fresh PBMC were cocultivated with 1×10^6 MT-2 cells, as described [6]. When absolute whole blood CD4 T cell counts of a given subject were less than 50×10^6 cells/l, 5×10^6 fresh PBMC were incubated with 1×10^6 MT-2 cells. The cultures were maintained at 37°C for 3 weeks and checked twice a week under an inverted microscope (× 4 magnification) for the presence of syncytia. Virus replication was measured by enzyme-linked immunosorbent assay for HIV-1 p24 antigen (ELISA, Vironostika, Organon Teknika) on culture supernatants

at the time point of syncytium formation or after a maximum of 3 weeks of coculturing.

HIV-1 biological cloning

Biological HIV-1 clones were isolated under limiting dilution conditions, as described [31,32], from all three patients harbouring SI as well as from a random selection of seven patients harbouring NSI. Graded numbers of patient PBMC (range $1–2 \times 10^4$ cells/well) were cocultivated in 96-well plates, with 10^5 phytohaemagglutinin (PHA)-stimulated PBMC from healthy blood donor volunteers. For each patient PBMC, 48 parallel micro-cocultures were performed. Fresh medium with interleukin 2 and PHA-stimulated donor PBMC were added every week. Virus replication, as reflected by the presence of HIV-1 p24 antigen in the culture supernatant, was determined by an in-house p24 capture ELISA. The SI capacity of the virus clones was determined by cocultivation of PBMC with MT-2 cells, as above. The proportion (F) of infected cells was determined according to the formula for Poisson distribution: $F = -\ln(F_0)$, where F_0 is the fraction of negative cultures. Only virus clones that were obtained from a dilution of patient PBMC that gave rise to progeny virus in not more than 33% of parallel cultures were considered clonal and used for further analysis. These clones were transferred to 25-ml tissue culture flasks containing 5×10^6 PHA-stimulated PBMC in 5 ml culture medium to grow virus stocks. For each patient, $0.9–6.6 \times 10^6$ PBMC (dependent on availability) was used as starting material for isolation of biological clones.

Cell lines and analysis of HIV-1 coreceptor usage

Human astroglioma U87 cell lines stably expressing CD4 and coexpressing CCR1, CCR2b, CCR3, CCR5 or CXCR4 chemokine receptors [33] were grown in Iscove's medium supplemented with 10% fetal calf serum, 5 μg/ml Polybrene, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml puromycin. The U87.CD4 single transfectant control cell line was grown in the same medium but without puromycin. As determined by FACS analyses, the CCR5 and CXCR4 U87.CD4 cotransfectants expressed the correct coreceptors. To determine coreceptor usage of biological HIV-1 clones, first the half-maximal tissue-culture infectious dose ($TCID_{50}$) was determined by titration on PHA-stimulated PBMC of a healthy blood donor expressing homozygous wild-type CCR5. Then, the determined $TCID_{50} \times 10^2$ was added in 0.2 ml volume to a 24-h culture of 10^4 cells of the various U87.CD4 cotransfectants in 96-well plates. The infected cells were incubated for another 24 h, washed with phosphate-buffered saline and 0.2ml fresh medium was added. At day 7, cells were detached by trypsinization and transferred to 24-well plates and cultured further. Supernatants harvested at day 7, 14 and 21 were analysed for the presence of HIV-1 by p24 ELISA. The p24-positive culture supernatants were considered indicative of the virus using the pertinent transfected coreceptor(s) for infection.

Finally, PHA-stimulated PBMC from a healthy blood donor with a homozygous 32 base-pair (bp) deletion in *CCR5* (*CCR5* Δ32) was inoculated with a $TCID_{50} \times 10^2$ dose of virus and the same procedure as above was used to analyse coreceptor usage other than CCR5 in a more physiological background.

RNA and DNA isolation and sequencing

The extractions of RNA from 100 μl plasma samples and 10 μl MT-2 coculture supernatants, as well as of DNA from clonal isolates, were performed by the guanidinium isothiocynate silica method, as described [30]. A region of 287 bp of the HIV-1 envelope, including the *gp120 V3* region, was amplified by nested polymerase chain reaction (PCR), as described [16,23]. The products were directly sequenced using the dideoxy chain termination method with sequenase (Amersham, Life Sciences, Little Chalfont, Bucks, UK), according to instructions from the manufacturer and analysed with an automatic sequencer ABI 370 A system (Foster City, CA).

Phylogenetic analysis

Alignments of sequences were performed manually. Phylogenetic analyses were carried out by using PHYLIP software packages, including the programs DNADIST, NEIGHBOR, a neighbouring-joining method under the Kimura two-parameter model [34,35], DRAWTREE, SEQBOOT (for 100 sets) and CONSENSE [36]. For comparison, representative consensus sequences of HIV-1 subtypes (A–H) from the Los Alamos data base (version 1998) were also included in the analyses.

Statistical analysis

The distribution of continuous variables (viral load, CD4 cell count) was compared between patients with different viral phenotypes (SI/NSI) using non-parametric methods (Mann–whitney U test). Proportions were compared using two-sided Fisher's exact test.

Results

Immunological and virological characteristics

Of the 48 patients with AIDS studied, the mean age was 35 years, both for females (20) and for males (28). The absolute CD4 T cell counts in whole blood varied between 2×10^6 and 194×10^6 cells/l with a median value of 54×10^6 cells/l. The absolute CD8 T cell counts in whole blood varied between 60×10^6 and 1989×10^6 cells/l; CD4/CD8 ratios ranged between 0.0 and 0.8. Viral loads varied between 4.26 and 7.08

Table 1. Characteristics of Ethiopian patients with AIDS infected with HIV-1 subtype C.

	Biological phenotype PBMC	
	SI (n = 3)	NSI (n = 45)
Males (%)	67 (2/3)	58 (26/45)
Mean age (years)	35	35
Stage IV[a]	67 (2/3)	84 (38/45)
CD4 cells, mean ($\times 10^6$ cells/l)	23*	78
CD8 cells, mean ($\times 10^6$ cells/l)	589	626
CD4/CD8 ratio, mean	0.03*	0.20
HIV load, mean ($\log_{10}$ copies/ml)	5.30	5.72
Coreceptor usage (%)[b]		
CCR1	0 (0/19)	0 (0/59)
CCR2b	0 (0/19)	0 (0/59)
CCR3	0 (0/19)	0 (0/59)
CXCR4	63 (12/19)	0 (0/59)
CCR5	5 (1/19)	98 (58/59)
CXCR4 + CCR3	26 (5/19)	0 (0/59)
CXCR4 + CCR5	5 (1/19)	0 (0/59)
Unknown[c]	0 (0/19)	2 (1/59)

[a]Percentage of patients at stage IV of WHO clinical definition of HIV-1 infection [25]. [b]Results of studies on 19 SI and 59 NSI clonal isolates in parentheses. [c]Unknown coreceptor used for successful *in vitro* infection of PHA-stimulated PBMC. *P = 0.04,Mann–whitney U test. PBMC, peripheral blood mononuclear cells; SI, syncytium-inducing phenotype; NSI, non-syncytium-inducing phenotype.

$\log_{10}$ RNA copies/ml plasma, with median of 5.59 log RNA copies/ml.

Cocultures of patient PBMC with MT-2 cells resulted in syncytium formation for only 3/48 (6%) subjects: patients 030, 074 and 079. The remaining 45/48 (94%) of PBMC from patients did not harbour any SI-inducing viruses, as determined by the MT-2 assay. As shown in Table 1, the mean whole blood CD4 count of the SI-positive patients was lower (23×10^6 cells/l) than that of the patients with NSI (78×10^6 cells/l) ($P = 0.04$). The viral loads for patients with NSI and SI HIV phenotypes did not significantly differ: 5.72 (SD 0.08) $\log_{10}$ RNA copies/ml for NSI versus 5.30 (SD 0.54) $\log_{10}$ RNA copies/ml for SI ($P > 0.05$). No significant correlation was detected between viral load and gender, age, clinical stage or any lymphocyte subset, including CD4 T cells.

Biological clones and coreceptor usage

In order to confirm the MT-2 results, HIV-1 biological clones were generated from PBMC of all three SI-harbouring patients and from PBMC of a random selection of seven patients carrying solely NSI HIV-1. Coreceptor usage of these clones was determined by *in vitro* infection of U87 cotransfectants as well as PHA-stimulated PBMC. A p24 ELISA was performed on culture supernatants to establish productive infection.

Table 1 shows the results from 78 biological HIV-1 clones derived from the above patients. The CCR5 coreceptor was used solely in 58/59 (98%) NSI clones; this usage was confirmed in PHA-stimulated PBMC from wild-type (wt/wt) and homozygous *CCR5* Δ32/*CCR5* Δ32 donor PBMC (data not shown). One NSI clone did not produce viruses (p24 negative) when tested on any of the chemokine receptor cotransfectants. However, this clone readily infected PHA-stimulated PBMC (p24 positive).

The coreceptor usage by SI clones was more heterogeneous: 12 clones used solely CXCR4, five were dual tropic for CCR3 and CXCR4, one was dual tropic for CCR5 and CXCR4 and one used CCR5 only. Except for the last, the CCR5 independence of these SI clones was confirmed by the fact that they could successfully infect homozygous *CCR5* Δ32/*CCR5* Δ32 PHA-stimulated PBMC (data not shown).

Sequencing of *gp120 V3*

Sequencing of *gp120 V3* was performed on PCR-amplified clonal DNA (cDNA) derived from all patient plasma samples. In addition, PCR-amplified cDNA from PBMC/MT-2 coculture supernatants and DNA from 23 biological HIV-1 clones derived from three patients harbouring SI HIV (patient 030, n = 6; patient 074, n = 10; patient 079, n= 7) was sequenced. The clones were also tested for coreceptor usage. The *gp120 V3* sequences are available from the authors on request and have been submitted to Genbank under accession numbers AF158843-AF158915.

In total, 73 *gp120 V3* sequences were determined, 20 from SI viruses and 53 from NSI viruses, and used to predict the structure of the viral protein gp120. The mean net charge of the predicted gp120 *V3* loop was significantly higher in SI sequences (6+) than in NSI sequences (3+) ($P < 0.001$). In NSI sequences, the predominant predicted motif of the apex of the V3 loop was GPGQ (51/53; 94%), while in SI sequences there was a positively charged residue replacing Q (H or R) for two of the three patients. Furthermore, the potential N-linked glycosylation site at position 6–8 of the V3 loop (relative to the first cysteine at position 1) was affected in most SI sequences and intact in NSI sequences. Either this glycosylation site was lost [in 13/20 (65%) of SI sequences and only in 1/53 (2%) of NSI sequences] or the site was shifted to position 5–7 [in 6/20 (30%) of SI sequences versus 0/53 (0%) of NSI sequences]. Finally, the positively charged amino acids at positions 11 and 25, documented to determine SI biological phenotype in HIV-1 subtypes A, B, D and E, were absent from the SI sequences obtained from the Ethiopian HIV-1 C subtype viruses. Instead, the combination of positively charged amino acids at positions 8 or 29 of the V3 loop appeared to correlate with 18/20 (90%) of the SI sequences and only 1/53 (2%) of the NSI sequences.

Phylogenetic analysis

Phylogenetic analysis was performed on all 73 HIV-1 *gp120 V3* sequences. Figure 1 is an unrooted phyloge-

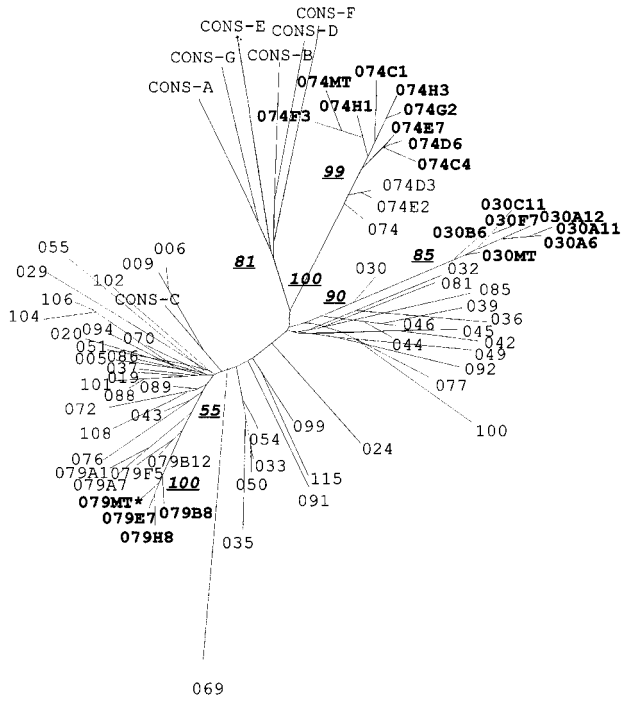

Fig. 1. Unrooted phylogenetic tree analysis of the 73 predicted amino acid sequences for the HIV-1 gp120 V3 region derived from 48 Ethiopian patients with AIDS. The patients are indicated in three-digit codes. Clonal isolates are in code suffices, e.g. A11, B6, etc. Sequences from SI clonal isolates and from MT-2 culture supernatants (suffix MT) are in bold. Los Alamos consensus sequences are abbreviated CONS- followed by subtype assignment. Numbers (bold and underlined) by the branches represent bootstrap values out of 100 replications. *For 47 plasma samples, the direct sequence data were unambiguous. Only the plasma sequence from patient 079 showed a mixed pattern. Therefore, the MT-2 culture supernatant sequence of 079 is shown instead.

netic tree in conjunction with Los Alamos 1998 consensus sequences for HIV-1 subtype A–H *gp120 V3* regions. From this figure it can be concluded that all HIV-1 *gp120 V3* sequences derived from our Ethiopian patients cluster with significant bootstrap values with the consensus C subtype sequence.

The sequences from patients 030 and 074 harbouring the SI virus cluster away from the bulk plasma sequences of the patients harbouring NSI virus, with bootstrap values of 90 and 100%, respectively. The sequences from the third patient (079) harbouring SI virus are more interwoven with the NSI bulk plasma sequences. For all three patients harbouring SI virus, the SI sequences cluster separately from the NSI sequences, with bootstrap values of 85–100%.

Discussion

HIV-1 phenotype variability plays an important role in the pathogenesis of AIDS. The presence of SI variants in > 50% of HIV-infected individuals has been associated for various HIV-1 subtypes with accelerated decline of CD4 T cells and progression to AIDS [3,7–9,30]. In this study, a remarkably low frequency of SI phenotypes was observed (only 3/48: 6%) in HIV-1 subtype C viruses in Ethiopian hospitalized patients with AIDS.

Biological phenotype was determined using a MT-2 assay [6] where the absence of syncytium formation is interpreted as indicating a NSI phenotype. Consequently any other factors that could also give this result were excluded as far as possible by increasing the input PBMC to 5×10^6 for subjects with CD4 cell counts of $< 50 \times 10^6$ cells/l whole blood, by confirmative p24 ELISA on coculture supernatants, by biological cloning of HIV-1 from 10 different patients and repeating the MT-2 assays on these clones and, finally, by checking coreceptor usage of these HIV-1 clones. All these experiments confirmed the finding that only three Ethiopian patients harboured SI viruses.

In general, it should be emphasized that the *in vitro* result of a MT-2 assay does not necessarily reflect the *in vivo* situation. Subjects harbouring NSI viruses by MT-2 assay can harbour CD4 syncytia, as determined by high-resolution microscopy and immunofluorescent staining [37]. However, given the above evidence, the under-representation of SI variants in Ethiopian patients infected with HIV-1 C-subtype compared with patients infected with other HIV-1 subtypes appears substantial. A low prevalence of SI viruses in subjects infected with HIV-1 subtype C has also been reported in other parts of the world [38]. HIV-1 subtype C viruses seem to be, in general, rarely of the SI phenotype; the reasons for this are not clear.

It could be speculated that expression of CCR5 is higher on cells of subjects living in countries where the HIV-1 C epidemic is present, thus providing less selective advantage to the virus for mutation to *CXCR4* tropic variants. CCR5 reportedly is preferentially expressed on activated and memory T cells and CXCR4 on naive T cells [39]. In the African context, because of highly prevalent infectious diseases, there is substantial immune activation [40–43]. This may result in persistent increased CCR5 expression and, thus, less selective force for HIV-1 to mutate away from its NSI phenotype. Along these lines, it has been reported that individuals infected with the HIV-1 B subtype have lower CCR5 expression levels parallelling delayed conversion from NSI to SI [44]. However, the HIV-1 subtype C epidemic is widespread in many countries, Ethiopia, Djibouti, India, China, Brazil, South Africa,

and most HIV-1 subtype C isolates from these countries are of NSI phenotype [23–25,45–49]. A recent study has confirmed the rarity of the SI phenotype in HIV-infected Caucasians in Djibouti [50]. It is highly unlikely that subjects from such diverse genetic and environmental backgrounds have identical immunological features favouring the persistence of HIV-1 NSI variants only.

Consequently, it might not be the host but merely the virus that determines the low emergence of SI variants in HIV-1 subtype C infections. It could be speculated that the structural constraints for HIV-1 C subtype viruses to change from NSI to SI phenotype are more demanding. In this light, it is remarkable that the amino acid positions 11 and 25, which determine NSI versus SI phenotype in many other subtypes [8,12,14–16], do not seem to have this role in C subtype viruses. Data from the present study suggest that two new amino acid positions (8 and/or 29) might correlate with HIV-1 subtype C SI phenotype. However, these data were collected on only three individuals and should be considered carefully and need further confirmation.

The HIV-1 NSI clonal isolates studied (except one, from patient 072) used solely CCR5 as a coreceptor and the SI clonal isolates (except one, from patient 030) used CXCR4, as reported elsewhere [36,51–53]. The exceptional clone from patient 072 might use a coreceptor other than CCR1, CCR2b, CCR3, CCR5 or CXCR4, since it successfully infected wild-type PHA-stimulated PBMC. The CCR5 usage of the exceptional SI clone from patient 030 might be reflected by its *gp120 V3* sequence, suggesting this clone represents a transitional stage of mutation from NSI to SI.

In conclusion, this study shows a remarkably low frequency of HIV-1 subtype C SI variants amongst Ethiopian patients with AIDS. In addition, CCR5 was identified as a major HIV-1 coreceptor in Ethiopians. To what extent the SI phenotypes of Ethiopian HIV-1 subtype C viruses correlate with distinct in *vivo* biological properties, needs further study.

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