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HIV-1 Coreceptor Tropism among Kenyans Under Highly Active Antiretroviral Therapy.

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Abstract

Despite the scale up of the use of combined highly active antiretroviral (ARV) therapy, many HIV-1 infected patients are still failing treatment in Kenya. In 2007, the Food Drug and Administration (FDA) approved the use of CCR5 antagonists in treatment experienced patients. CCR5 antagonists work by inhibiting the entry of HIV-1 that uses CCR5 as a coreceptor to gain entry into cells. CCR5 59029 A/G (promoter region—rs1799987) is a polymorphism that leads to the upregulation of the expression of the CCR5 protein thereby affecting the rate of HIV-1 infection. The use of these CCR5 antagonists in Kenya is limited partly because of minimal data on host genetics and coreceptor tropism among HIV-1 infected patients. In this study, we aimed at determining the prevalence of CCR5 tropic variants and CCR5 59029AG promoter polymorphism known to influence HIV-1 infection. We sequenced the V3 region of the *env* gene and inferred the HIV-1 tropism using clonal model of Geno2Pheno algorithm (FPR= 5%). Also, we assessed the frequency of the CCR5 promoter polymorphisms among the patients by sequencing the polymorphic region of the CCR5 promoter. Majority of the patients (77.27%) had R5 tropic viruses whereas 22.73% of the study subjects had detectable CCR4 using viruses. The frequencies of the CCR5 59029 AA, AG, and GG genotypes were 14 (31.82%), 9 (20.45%) and 21(47.73%), respectively. Taken together, these results indicate that CCR5 antagonists could have potential therapeutic effects in the clinical management of HIV-1 among the infected patients in Kenya.

Key words: CCR5 antagonists, CCR5 59029AG, HAART, HIV-1, Polymorphism, Tropism

Background information

Acquired Immunodeficiency Syndrome (AIDS) is one of the major diseases that is responsible for the loss of millions of lives worldwide(Unaids, 2013). HIV, the causative agent of AIDS, uses chemokine receptors (cysteine-cysteine receptor 5 [CCR5] and/or cysteine-X-cysteine receptor 4 [CXCR4]) as coreceptors to enter CD4 expressing cells(Alkhatib, 2009). CXCR4 utilizing viruses are referred to as R4 tropic whereas CCR5 tropic viruses are known as R5 tropic viruses. Dual tropic viruses use both CXCR4 and CCR5 coreceptors to infect cells.

To inhibit HIV replication, a number of HIV-1 antiretroviral therapies have been designed(Cohen et al., 2013). To date, 29 individual antiretroviral drugs from six drug classes have been approved by the U.S. Food and Drug

Administration (FDA) to be used in the treatment of HIV-1 infection, including protease inhibitors (PI), nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitors (INI), fusion inhibitors (FI), and entry inhibitors (EI)(World Health Organisation, 2013). Majority of these classes of ARVs act on the various steps of the virus life cycle except the entry inhibitors which targets the inhibition of HIV entry through binding to the coreceptors. Up to date, only Maraviroc (CCR5 antagonist) has been approved by Food Drug Administration (FDA) for use as a CCR5 inhibitor whereas several others are under development(Sayana & Khanlou, 2009). These host-target ARV drugs promise a good therapy alternative especially for patients who are failing treatment due to virus resistance.

Cases of drug resistance among HIV-1 infected patients have been reported in Kenya(Hassan et al., 2013, 2014; Kebira & Khamadi, 2011; Kiptoo et al., 2013; Lel, Ngaira, Lihana, & Khamadi, 2014; Sigaloff et al., 2012). Although host targeted ARVs are being used elsewhere to counter the effects of drug resistance, their use in Kenya is limited partly because there's minimal data on viral tropism and host genetics. It is highly recommended that we determine co-receptor tropism before initiating treatment with host targeted ARVs because they have selective effect on viral populations. In this study, we employed genotypic assay to asses HIV-1 coreceptor use. Also, we determined the frequency of the CCR5 59029 AG promoter polymorphism that is known to affect rate of expression of the CCR5 coreceptors and subsequently influence disease progression.

Methodology

Study population

Individual patients aged 22-61 years were enrolled between April and August 2013 from 6 comprehensive clinical care facilities in the counties of Homa Bay, Kisumu, Kajiado, Nakuru, Kiambu and Malindi, which represented 5 geographical provinces of Kenya. All study subjects provided written informed consent.

Laboratory Procedures

Five milliliters of venous blood was drawn into EDTA vacutainer tubes from each patient and centrifuged at 3000rpm for 10 minutes to separate plasma from cells. Peripheral blood mononuclear cells (PBMCs) were obtained from plasma-free blood by lysing the blood with 0.84% ammonium chloride.

RNA extraction and amplification of the Env gene.

RNA was extracted from plasma of patients with viral load above 1000 RNA copies/ml using QIAamp Viral RNA Mini Kit while DNA was isolated from PBMCs of patients with undetected viral load using QIAamp DNA Mini Kit. All protocols were strictly as outlined in manufacturer's manual. The RNA samples were subjected to One-Step RT-PCR amplification, using envelope primers M5- 5'-CCAATTCCCATACATTATTGTGCCCCAGCTGG -3' (forward) and M10-5'-CCAATTGTCCCTCATATCTCCTCCTCCAGG -3' (reverse). PCR conditions were reverse transcription at 50^{o}C for 30 minutes, denaturation at 95^{o}C for 15 min and 38 PCR cycles of denaturation at 94^{o}C for 30sec; annealing for 45sec at 58^{o}C and extension at 72^{o}C for 1min. First-round PCR for DNA template followed similar

conditions with exception that the RT step was excluded and HotStar Taq DNA polymerase was used instead of RT enzyme. Nested PCR was carried out using HotStar Taq polymerase under conditions similar first round PCR with the following exceptions: initial denaturation at 95^{o}C for 5 min; 38 cycles of denaturation at 95^{o}C for 30s, annealing at 56^{o}C and extension at 68^{o}C for 45s and final extension at 68^{o}C. Primers for nested PCR were M3_5'- GTCAGCACAGTACAATGCACACATGG-3' (forward) and M8_5'- CCTTGGATGGGAGGGGCATACATTGC-3' (reverse). PCR amplification strategy targeted a 546 base pairs envelope C2V3 corresponding to nucleotides 6975–7520 on the HIV-1 HXB2. ***Genomic DNA extraction and genotyping of the CCR5 gene***

Genomic DNA was extracted from PBMCs using the QIAmp DNA mini kit (Qiagen, Germany), according to the manufacturer's instructions. The concentration of the isolated genomic DNA was measured using a spectrophotometer to check the quality and stored at -20^{o}C until use. The isolated DNA was then amplified by polymerase Chain Reaction (PCR). Each PCR reaction had a final volume of 25 µL PCR reaction mixture containing 2 µL of DNA of ~200ng/µL, 0.4mM dNTP mix, 2.0mM $MgCl_2$, 0.75 Units of Amplitaq Gold DNA polymerase, 1X Amplitaq Gold buffer, nuclease free water (Applied Biosystems, Foster City, CA, USA) and 0.4 µM of the forward and reverse primer. The forward and reverse primer sequences used for the amplification of polymorphic CCR5 regulatory region were 5- TGGGGTGGGATAGGGGATAC -3 and 5- TGTATTGAAGGCGAAAAGAATCAG -3 respectively. Each amplification reaction consisted of an initial denaturation of 95^{o}C for 15 minutes, 40 cycles at 95^{o}C for 30 sec, 58^{o}C for 30^{o}C and 72^{o}C for 1 minute and a final extension of 72^{o}C for 10 minutes. The CCR5 segment produced using the above mentioned primer was 498 bp in length.

The PCR products were then separated using 1.6 % agarose gel stained with ethidium bromide and visualized under UV light followed by purification using the Qiagen PCR purification kit. Purified products were sequenced using standard Big-dye chain terminator chemistry.

Sequence assembly and analysis

Sequences were manually inspected, pairwise aligned within the BioEdit sequence analysis using Clustal W. At least 2 reference sequences per subtype were included in the alignment of the HIV-1 env sequences. All the CCR5 promoter sequences were also aligned with the reference sequence U95626 using CLUSTALW installed in Bioedit software(Hall, 2001). The presence of SNPs was analyzed and heterozygosity determined by manually inspecting the chromatograms. For the heterozygous individuals, multi-colored peaks for both alleles were observed and were shorter in height than those of homozygous individuals.

Ethical statement

This study commenced after getting approval from the Kenya Medical Research Institute Scientific and Ethical Committees SSC No. 2477. Written informed consent was obtained from each participant prior to sample collection.

Statistical Analysis

Data analysis was carried out using the statistical package for social studies (IBM Corp. Released, 2011) Direct gene counting was used to determine the frequencies of the CCR5 genotypes and alleles.

Results

Tropism genotyping by Geno2Pheno tool.

HIV-1 tropism was identified through genotype analysis of the HIV third hypervariable loop (V3) of the *env* gene using Geno2pheno online tool(Sing, Beerenwinkel, & Kaiser, 2005). Since the samples were from therapied-patients, a clonal model of false positive rate [FPR] cut off of 5% was used as suggested. Also, to minimize the number of false predictions of CXCR4 tropic sequences as CCR5 tropic, tropism was inferred using 11/12 rule to predict an CXCR4 tropic sequence otherwise they were considered R5 tropic virus.

Table1: Coreceptors Usage.

	Coreceptor Usage N (%)
Coreceptor	
R4	10(22.73%)
R5	**34(77.27%)**

Table2: CCR5 59029 AG Tropism data.

GenBank Accession numbers

The HIV-1 sequences reported in this study have been deposited in the GenBank and assigned the following Accession numbers: KM853037- KM853149.

Prevalence of CCR5-59029 A/G Polymorphism among Kenyans under HAART

Genotyping of the CCR5 59029 A/G polymorphism was performed in 44 patients infected with HIV. The frequencies of the AA, AG, and GG genotypes were found to be 14 (31.82%), 9 (20.45%) and 21(47.73%), respectively.

	Gender N (%)		Total
	Males	Female	
CCR5- 59029 Allele			
AA-Homozygous	3 (21.43)	11 (78.57)	14 (31.82)
GG-Homozygous	4 (44.44)	5(55.56)	9 (20.45)
AG-Heterozygous	9(42.86)	12(57.14)	21 (47.73)

Table 2: Prevalence of CCR5-59029 A/G Polymorphism among Kenyans under HAART

Discussion

CCR5 antagonists are the latest class of antiretroviral drugs that are under development, with maraviroc being the only CCR5 antagonist that has been approved by FDA for clinical use. It is necessary that before their use, coreceptor usage should be determined. Recently, we established that subtype A viruses are still the predominant HIV-1 strains in local circulation in the Kenyan population(Kitawi et al., 2015). To the best of our understanding, there is little data on coreceptor usage in a population where HIV-1 subtype A is predominant. In this study, sequence of the partial env gene encompassing the V3 region of forty four samples from Kenya was analyzed. Subsequently, we estimated the frequency of HIV-1 coreceptors tropism among HIV-1 infected patients under HAART. The study population involved treatment experienced Kenyans. Majority of the study population (77%) were found to be infected with the R5 tropic viruses, the rest were infected with R4 tropic variants. None of the study subjects had a dual infection (R4/R5 tropic variants). These results were consistent with other results elsewhere involving HIV-1 non subtype B viruses. We could not however determine whether antiretroviral treatment creates an environment for the emergence of CXCR4 tropism.

We also determined the frequency of CCR5_59029AG polymorphism that is known to influence the rate of disease progression. CCR5 59029A/G is an A/G transition at base pair 59029 in the CCR5 promoter that has been shown to influence AIDS pathogenesis(Kostrikis et al., 1999). HIV-1 infected individuals with the CCR5-59029G/G genotype progress more slowly to AIDS than those with the A/A genotype. The frequencies of the AA, AG, and GG genotypes were found to be 14 (31.82%), 9 (20.45%) and 21(47.73%), respectively among the study subjects.

To the best of our understanding, this is the first study focusing on the prevalence of HIV-1 tropism among therapied-patients in Kenya. These results may therefore assist in the therapy guide among Kenyans living with HIV.

Conclusion

Our data on the HIV-1 coreceptor tropism in Kenya and the prevalence of CCR5 promoter polymorphism is important for therapy planning especially on the use of CCR5 antagonists as options for HIV patients in Kenya. The existence of R4 tropic viruses should seriously be considered when decisions are made about regimen change for therapy-experienced individuals. This means that HIV-1 coreceptor usage should be screened before initiation of any chemokine receptor CCR5 antagonists in therapy experienced Kenyans living with HIV. Also

the high frequency of the CCR5GG genotype among HIV-1 infected individuals is noteworthy.

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