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Determination of the Existence and Distribution of HIV-I Chemokine Co-Receptor 5 Polymorphism in a Sampled Population from Kenya

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Abstract

Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) is a major public health problem, socio-economic burden and a serious threat to development. Entry of human immunodeficiency virus type 1 (HIV-1) into target cells requires the binding of the external envelope glycoprotein *gp 120* to both the CD4 molecule and one of several chemokine receptors, recently discovered to function as co-receptors. T –cell line tropic HIV-1 strains utilize the α-chemokine receptor CXCR4, whereas the β-chemokine receptor 5 (CCR5), which is expressed on monocytes/macrophages, T cells and granulocyte precursors, is the key co-factor for macrophage-tropic HIV-1 strains, which predominate during the asymptomatic phase of infection. A thirty two–base pair (bp) deletion mutation (Δ 32) within the second extra cellular loop-encoding region of the CCR5 gene, which results in a truncated, non-functional protein, has been associated with relative resistance to HIV -1 infection and slower progression to acquired immunodeficiency syndrome (AIDS). Specifically, Δ32/Δ32 homozygotes are protected against acquisition of HIV-1 by the mucosal route despite high risk exposure, whereas disease progression among CCR5/Δ32 heterozygote occurs more slowly. In this study, the status of the CCR5 gene polymorphism in Kenyan population was investigated in an attempt to explain the differences in HIV prevalence in different parts of the country. To determine this, 200 samples were collected from the 8 provinces of Kenya, that is, 25 samples per province, some of which were positive for HIV-1. Twenty-five samples were randomly selected from a batch of 250 per province, that is, every tenth sample. The samples were collected from HIV screening centers, district and provincial hospitals. Peripheral blood mononuclear cells (PBMC) were extracted from whole blood. Genomic deoxyribonucleic acid (DNA) was then extracted from PBMC. A targeted region of the CCR5 gene flanking the 32bp deletion was amplified by polymerase chain reaction (PCR) using CCR5 specific primers. All the PCR amplicons were then analyzed by gel electrophoresis. The results showed that CCR5-Δ 32 mutations do not exist in the Kenyan population. Samples were then randomly selected 4 samples per province and sequenced. This was done to determine the genotype of the PCR products that were amplified. After ClustalW analysis of the sequences generated, it was seen that CCR5 gene is not highly conserved in the Kenyan population, as there were amino acid differences between the sequences analyzed suggesting that CCR5 gene in Kenyan population is highly polymorphic. From this study, it was concluded that CCR5-Δ 32 mutations do not play any role in HIV-1 susceptibility in the Kenyan population. This is because this mutation does not exist in the Kenyan population as per the samples analyzed. The differences in prevalence of HIV in different parts of the country may be due to cultural practices, religious backgrounds, socio-economic status and other intrinsic genetic factors.

Keywords: HIV/AIDS, chemokine receptors, CCR5/Δ32

1. Introduction

Acquired immune deficiency syndrome (AIDS) is a collection of symptoms and infections resulting from the depletion of the immune system caused by infection with the Human Immunodeficiency Virus (HIV), which is a retrovirus belonging to the lentivirus family of viruses that cause slow progressing diseases (Colebunders *et al*., 1997). The immune deficiency results from loss of the CD4+ T cells that are essential for both cell-mediated immunity and humoral immunity. Acquired immune deficiency syndrome (AIDS) symptoms are mostly opportunistic infections that can be easily treated in healthy people (Colebunders et al., 1997). The disease has four stages: the first stage is asymptomatic. The second stage includes minor mucocutaneous manifestations and recurrent upper respiratory tract infections. Chronic and pulmonary tuberculosis is seen in stage three. Stage four includes the toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi or lungs and kaposis sarcoma (Colebunders *et al*., 1997). Transmission routes of HIV are sexual, blood-to-blood products and mother to child transmission (Stewart et al., 1985).

Human Immunodeficiency Virus (HIV) was first recognized in 1981 among homosexual men in United States of America and heterosexual women who had a severe immunodeficiency (Harris *et al.*, 1983). Globally, the leading mode of transmission is heterosexual. Human Immunodeficiency Virus (HIV) challenge varies geographically. About 96% of people with HIV live in the developing world, mostly in sub-Saharan Africa and the Caribbean (UNAIDS/WHO, 2005). An estimated 5 to 6 million people in low and middle-income countries will die in 2007 if they do not receive antiretroviral treatment (ART; UNAIDS/WHO, 2005). By the end of 2005, it was only 7% who needed ART were receiving it (UNAIDS/WHO, 2005). In some parts of Africa, one-third of all pregnant women have HIV/AIDS. Adult infection rates in sub-Saharan Africa have reached as high as 37% in Botswana and 39% in Swaziland (UNAIDS/WHO, 2005). In the Russian Federation, more than 90% of the estimated one million people living with HIV were infected through intravenous drug use, but they make up only 13% of those receiving antiretroviral therapy (UNAID, 2005).

The existence of uninfected individuals despite persistent exposure to HIV suggests the existence of genetic factors, which play a role in host susceptibility (Just *et al*., 1995). Although specific genes of the human leucocytes antigen (HLA) system were found in certain groups of frequently exposed uninfected sexual workers in Kenya and Thailand, it was the discovery of a CC chemokine receptor polymorphism that for the first time unequivocally linked a single gene to host susceptibility (Just *et al*., 1995).The Δ 32-allele incidence is high in Caucasian populations but is thought to be low in the African and Asian populations (Samson *et al*., 1996). The homozygous Δ 32 conditions produce a truncated receptor, which is retained and degraded in the endoplasmic reticulum and therefore not expressed on the cell surface (Lui et al., 1996). Individuals heterozygous for the deletion are not resistant to HIV infection, but once infected, have a slower progression towards AIDS (Eugen et al., 1997; Meyer et al., 1997; Dean et al., 1996; Garred et al., 1996). This prolonged AIDS-free survival is associated with a slower decline in the CD4+ T-cells and a lower viral load (Katzenstein *et al.*, 1997). However, at the onset of AIDS, heterozygosity offers no advantage over the wild type condition (Michael et al., 1997; Smith et al., 1997; Garred *et al*., 1997).

Even though the prevalence of the Δ 32 allele in certain high-risk sero-negative populations has been reported, only 4% of all the highly exposed HIV-sero-negative persons are homozygous carriers of the deletion (Huang *et al.*, 1996). Although the allele frequency of the defective CCR5 gene in high incidence regions like sub-Saharan Africa and South East Asia is low, it has been argued that the global impact of this type of resistance is of minor importance (McNicholl *et al*., 1997). In the Kenyan population, whether this condition exists is unknown. Since individuals homozygous for the CCR5 deletion are not phenotypically abnormal and have a normal life expectancy, the CCR5 receptor could be a suitable target for therapeutic intervention (McNicholl *et al*., 1997). The strategy would be to block the CCR5 receptor, therefore reducing the chances of macrophage-tropic strains of HIV that use CCR5 as its co-receptor but not T-tropic strains of HIV which use CXCR4 as its co-receptor. It is important to determine the status of the CCR5 gene mutations in Kenyan population so as to determine if CCR5 Δ-32 mutations influence HIV-1 susceptibility and distribution in the Kenyan population. Prevalence of HIV in Kenya has been observed to vary from region to region with the highest prevalence in Nyanza province and the lowest in North Eastern province (Central Bureau Statistics CBS, Kenya Demographic and Health Survey 2003, Populations Projections, and NASCOP).

2. Materials and Methods

2.1Separation of Peripheral Blood Mononuclear Cells (PBMC) from Whole Blood

Peripheral blood mononuclear cells (PBMC) were separated from whole blood as described by Chomczynski *et al*., (1997). Briefly, 5 ml of whole blood was added to a falcon tube containing 10 ml of 0.84% ammonium chloride and vortexed, and incubated at $37C^0$ for 5-10 minutes. The mixture was then span at 1500 rpm for 10 minutes at room temperature and the supernatant discarded. Another 10 ml of 0.84% ammonium chloride was added to the pellet and the procedure repeated three times until the PBMC pellet appeared white. The supernatant was then discarded and 1ml of 0.84% ammonium chloride was added to the falcon tube with the pellet and mixed with a pipette. The pellet was then drawn into a 1.5-ml Eppendorf tube and span at 1500 rpm in a microfuge at room temperature. The supernatant was then pipetted off to recover the PBMC, which appeared as white pellet. In case there was still a red colouring of the PBMC, the red portion was sucked off with the aid of a vacuum pump.

2.2 Genomic DNA Extraction

Deoxyribonucleic acid (DNA) was isolated from PBMC according to the method described by Chomczynski *et al.,* (1997). Briefly, to the PBMC pellet extracted as described above, 500 µl of DNAzol genomic DNA extraction reagent (Gibco BRL®, USA) was added. The pellet was dissolved completelyby pipetting the reagent-pellet mixture up and down several times. Two volumes (1000 µl) of chilled (cooled to 4^0C) absolute ethanol was added to the dissolved pellet and mixed gently. This was centrifuged at 3000 rpm in a microfuge at 4^0C for fifteen minutes. The supernatant was discarded and1000 µl of 70% ethanol added to the pellet and vortexed thoroughly. The sample was centrifuged again at 3000 rpm in a microfuge at 4^0C for fifteen minutes and the supernatant discarded. The pellet was dried in a safety cabinet at

room temperature. The DNA pellet was resuspended in 100 µl of distilled DNase and RNase free water and stored at -20°C till further use.

2.3Cystein-Cystein linked chemokine receptor 5 (CCR5) Genotyping by Polymerase Chain Reaction (PCR)

Genotyping of CCR5 polymorphism was carried out using one-step Polymerase Chain Reaction (PCR) with primers flanking the region containing 32-bp deletion as described by Martinson *et al*. (1997). The targeted region of the CCR5 gene flanking the 32-bp deletion was amplified using a pair of oligonucleotide primers that are able to differentiate between mutant and natural CCR5 alleles without restriction endonuclease digestion. Each PCR was conducted in a final volume of 50 µl containing 0.2-0.5 µg template DNA, 1x PCR buffer, 200 µM each dNTP, 1 mM $MgCl_2$, 1.25 U DNA polymerase (Thermus aquaticus; Perkin-Elmer Corporation, Norwalk, CT-USA), and 50 nM each primer under sterile conditions. Thermocycling conditions consisted of 45 cycles with an initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The mutated and unmutated alleles were expected to appear as 147-bp and 179-bp amplicons, respectively (Liu *et al*; 1996; Huang *et al*; 1996; Samson *et al*; 1996; Dean *et al*., 1996; Zimmerman *et al*., 1997).

2.4 Gel Electrophoresis

Products of PCR were analyzed by conventional agarose electrophoresis. This was used to resolve the PCR products (Sambrook *et al*., 1989). The agarose gel was prepared using 1X Tris Borate EDTA (TBE) where 0.4 grams of 3% agarose powder was placed in a 125ml flask. 50 ml of 1 X TBE was then added to the flask and mixed completely by staring. The solution was then heated in a microwave to dissolve all the agarose powder. The solution was the left to cool while starring gently for sometimes until it was about heavy/thick. The combs were then placed in the slots on the side of the gel tray and the solution poured on the gel tray.

The gel was then left to solidify after which the combs were removed after creating wells on the gel for loading samples. The gels with the wells were then placed in the gel tanks ready for loading the samples. The first well of the gel was loaded with 3µl of 100 base pair molecular weight marker while the consecutive wells were loaded with the samples. The samples were mixed with gel loading buffer before loading into the wells on the gel, that is, 3µl of the loading dye (Bromophenol Blue) was first placed on a parafilm and 5µl of the samples added. The gel tank was then closed and plugged in the electrodes to the power supply and turned on. Electrophoresis was done at a constant voltage of 80-120 volts/cm using a Bio-Rad model 200/2-power supply source for 30-45 minutes. Once the electrophoresis was completed, the gel was stained with 0.5 µg/ml ethidium bromides for visualization using an ultra violet (UV) spectrophotometer. The location of the mutated and unmutated alleles on the gels was determined by direct examination of the gel and the size estimated by comparing with molecular weight markers loaded alongside the sample under UV light (Sharp *et al*., 1973). The photographs of the gels were then taken using Polaroid camera.

2.5 Validation of CCR5 Genotyping Assay using DNA Sequencing

To determine the genotype of CCR5 genes, randomly selected PCR amplicons, (4 per province), were sequenced using Bigdye® kit (Applied Biosystems USA). In this system, fluorescently labeled dyes that label and distinguish the four bases (ACTG) are attached to these bases extension products in DNA sequencing reactions as follows; Red labels Thymine bases, blue labels Cytosine bases, black labels Guanidine bases and green label Adenine bases.

To incorporate these dye labels, ether 5'-dye labeled primers or 3'-dye labeled dideoxynucleotide terminators were used. The enzyme used for primer extension is AmpliTaq® polymerase (PE Biosystems, Foster City, California, USA) and the template is the PCR product that was to be sequenced. The dyes and the template were then used to perform sequencing PCR by adding 3µl of 5x buffer and 2 µl of Bigdye and 1.5 µl of primers for each sample as previously described (Takehisa *et al*., 1999). The sequencing PCR conditions were as follows: 96^0C for five minutes followed by 25 cycles of 96^0C for 10 seconds, 50^0C for 5 seconds and 60^0C for 4 minutes (Carr *et al*., 1998; Kostrikis *et al*., 1998). The primers attaches to the denatured template during the annealing step. The temperature is raised to 60^0C and the Taq polymerase extends the primer, incorporating ddNTPs that are fluorescently labeled that stop the extension reactions to randomly generated fragments that differ in length by one base.

The sequencing PCR products are labeled amplicons which were then precipitated by adding 2µl of 3M, Sodium acetate (PH 4.6) and 50µl of absolute ethanol to each sample in a 1.5 ml Eppendorf tube. To precipitate the DNA, the Eppendorf tubes containing the samples were left at room temperature for 15 minutes and then span at maximum speed for 20 minutes and the supernatant aspirated from each tube. 250 µl of 70% ethanol was then added to each tube and vortexed briefly and then span again for 10 minutes at maximum speed and the supernatant aspirated from the tubes. The samples were completely dried in a vacuum centrifuge for 10-15

minutes and 25 µl of template suppression reagent (TSR) added to each sample pellet and vortexed thoroughly. They were heated for 2 minutes at 95^0C and then chilled on ice and vortexed thoroughly and span down briefly in a microcentrifuge. The sample were transferred to sequencing tubes and then loaded onto the automated sequencer in the instruments autosampler and sequenced by electrokinetic injection according to the instruction given in the operational manual (ABI 3100, Applied Biosystems, Palo Alto, California, USA).

2.6 Sequence Analysis by ClustalW

Once the sequences were generated, they were analysed using ClustalW software (Higgins *et al.,* 1994; http://align.genome.jp/).The base sequences generated were first translated into amino acids before being analysed. To translate the base sequences into amino acids, translation for publication software was used (http://bioinfo.hku.hk/services/analyseq/cgi-bn/forpubin.pl). This programme translates the base sequences into amino acids so that they can be aligned by clustalW so as to determine the level of conservation of the CCR5 gene. (http://bioinfo.hku.hk/services/analyseq/cgi-bn/forpubin.pl). Once the amino acids sequences were generated, they were aligned using ClustalW (Higgins *et al.,* 1994).

2.7 Determination of Polymorphism of CCR5 Gene

The amino acids generated were then used to generate a phylogenetic tree using Tree View program (Page, 1996). The phylogenetic tree was used to determine polymorphisms in the CCR5 gene by grouping the amino acid sequences into different clusters based on similarity.

3. Results

3.1 Gel electrophoresis

After PCR products were analysed using gel electrophoresis, this study revealed that the 32 bp mutation was absent in all the samples analyzed since all the bands were of 179 molecular weight and no band were of 147 bp as would have been the case incase of the mutation (Figure 1). When DNA sequencing was done, the sequences showed that the order of the bases was different in all the samples analyzed. The sequences also revealed that although the base sequences were different in all the samples, some regions of the CCR5 gene in all the samples had same order of the bases (Figure 2).

When all the samples that were sequenced were translated into amino acids, the results revealed that the amino acids in the samples were different in the entire samples analyzed (Figure 3).

Figure 1.Gel electrophoresis of polymerase chain reaction (PCR) product targeting amplification of mutated gene. Extracted DNA from PBMC samples were amplified with primers (Forward and Reverse) targeting CCR5 flanking 32 bp deletions.

3.2 DNA sequencing

Sample 1

>:TCCCTTCGGGCGTATTTCCCTCAGCAGTATCAATCGTGGAAGAATTCCAGACA**TTAA**AGA
TAGCCATCTTGGGGCTTGGTCCTTGCCAGCATGCTTTGGCCAGTGGCCAAACTTGCTTACCT
CGGGAATCCTAAAA//

Sample 2

>:CACCTTGCGAGCA**TTAA**TTTTCCCTACAGCCAGATATCAAATTCTTGGAAGCAATTTCCA
GACATTAAAGATAGTCATCTTTGGGGCTTGGTCCTTGCCAGCATGCCTTGTCAATGGCTCTC
TCTTGCTATACCTCGGGGAAATCCTTAAAAAACGTGC//

Sample 3

>:TCCCTTGCGGTCTATTTTCCTCAGCAGTATCAATTCTTGGAAGAATTCCAGACA**TTAA**AG
ATAGTCATCTTTGGGGCTTGGTCCTTGCCAGCATGCCTTGACGATGGCTCAATCTTGCTTAC
CTCGGGGGAAATCCTT//

Sample 4

>:TCACCTTGCGAGCATCTTAATTTTCCTCTACGAGCTCAAGTATCAATTCTTGGAAAGAAA
TTTCCCAGCACTA**TTAA**AGGATAGTCGATCTTTGGGGCTTGGTCCTT
TGCTTTGTCCATGGCTCAATCTTGCTTACCTCGGGGAA
ATCC//

Figure 2a**.** Sequenced data of the samples from North Eastern province. PCR products were sequenced using DNA sequencing PCR with dNTPs labeled with different fluorescence dyes.

Sample 1

>:CCCTCAGCACATATTTCCATTCCAGTCAGTACAATCTGGAAGAATTTCCAGACA**TTAA**AG
ATAGTCATCGTTGGGGCTTGGTCCTTGCCGCATGCGTTGACCATGCGCTCTTCTTGCTTTCC
TC//

Sample 2

>:CCCTTCAGCGCGTAATTTCCATCAGTCAGTATCAAATTCTGGAAGAATTCCAGACA**TTAA**
AGATAGTCATCTTTGGGGCTTGGTCCTTGCCTCATGCTTTGTCAATGGCTCAATCTTGCTTA
CCTCGGGAAACCCTTAAAAA//

Sample 3

>:CCCTTCAGACTTATTTTCCTCAGCAGTATCAATCGTGGAAGAATTCCAGACA**TTAA**AGAT
AGTCATCTTTGGGGCTTGGTCCTTGCCAGCATGCTTTGTCCATGAAACAATCTTGCTTACCT
CGGGAAATCCTTAAAAAT//

Sample 4

>:CCGTGCGGGGGGTTAAATTTTCCTATCCAGCCAAGATATCGAATTCTTGGCAAGAATTTC
CAGACCA**TTAA**AGAATAGCTCTATCGTTGAGGGCTTGGCCCTTGCCAGCATGCTTTGACAA
TGAAACAATCGTGC//

Figure 2b: Sequenced data of the samples from Nyanza province. PCR products were sequenced using DNA sequencing PCR with dNTPs labeled with different fluorescence dyes.

Key:

T=Thymine bases, **C=**Cytosine bases, **G=**Guanine bases and **A=**Adenine bases

3.3 Translation to amino acid

Sample 1
>: H L A S I N F P Y S Q I S N S W K QF P D I K D S H L W G L V L A S M P C Q W L S L A I P R G N P * K T C//

Sample 2

>: S L A V Y F P Q Q Y Q F L E E F Q TL K I V I F G A W S L P A C L D D GS I L L T S G E I L//

Sample 3

>: S P C E H L N F P L R A Q V S I L GK K F P S T I K G * S I F G A W S LP A C F V H G S I L L T S G K S//

Sample 4

>: H L Q R Y F L Q A V S N S W K N S RH * R * P S L G L G P C R M R * S W L N L A Y L G E I//

Figure 3a. Amino acid sequence data of the samples from North Eastern province. The base sequences were translated into amino acids using translation for publication software.

Sample 1

>: P S A H I S I P V S T I W K N F Q T L K I V I V G A W S L P H A L T M RS S C F P//

Sample 2

>: P F S A * F P S V S I K F W K N S R H * R * S S L G L G P C L M L C Q WL N L A Y L G K P L K//

Sample 3

>: P F R L I F L S S I N R G R I P D I K D S H L W G L V L A S M L C P * N N L A Y L G K S L K//

Sample 4

>: P C G G L N F P I Q P R Y R I L G K N F Q T I K E * L Y R * G L G P C Q H A L T M K Q S C//

Figure 3b. Amino acid sequence data of the samples from Nyanza province. The base sequences were translated into amino acids using translation for publication software.

Key:

A=Alanine, R=Arginine, N=Asparagine, D=Aspartic acid,
C=Cysteine, Q=Glutamine, E=Glutamic acid, G=Glycine, H=Histidine, I=Isoleucine, K=Lysine, L=Leucin, M=Methionine, F=Phenylalanine,
P=Proline, S=Serine, T=Threonine, W=Tryptophan, Y=Tyrosine,V=Valine and * =Stop Codon.

3.4 Alignment using ClustalW software

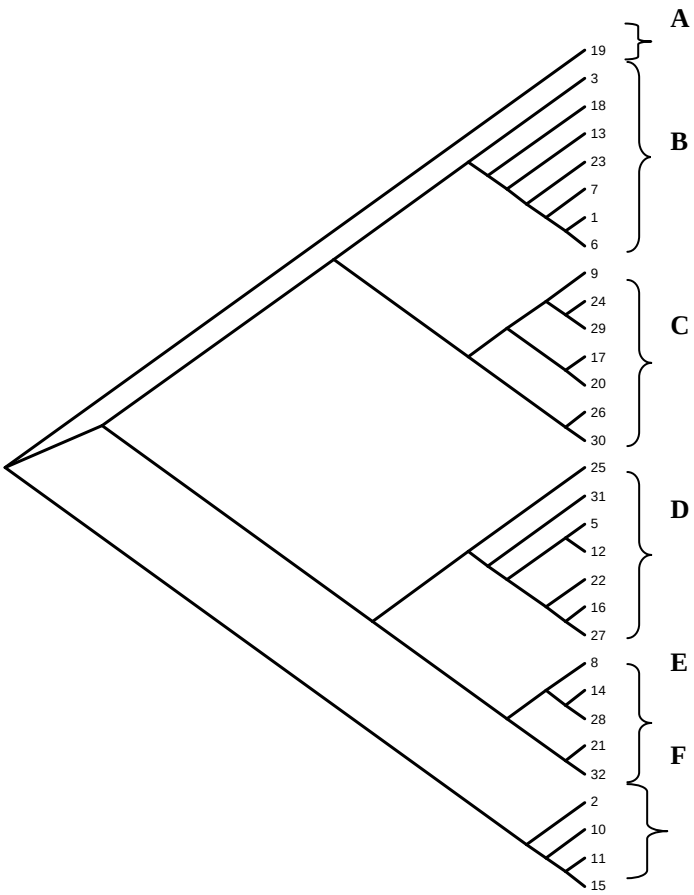
In this study, all the sequences that were generated were aligned using ClustalW software (Higgins *et al*., 1994). This was aimed at identifying the conserved sequence regions of the CCR5 gene in the Kenyan population. The results showed that CCR5 gene is not highly conserved in the Kenyan population. This is because; each sequence showed different amino acids as shown by different colours in the alignment suggesting that each sample has different amino acid sequence thus different from each other (Figure 4). From the alignment, it was also seen that no amino acids in all the columns were identical in all the samples (Figure 4). Although CCR5 was found not to be highly conserved, it was revealed that the gene had some conserved and semi-conserved regions as underlined suggesting that there are some regions in the sequences that are similar in the samples (Figure 4).

Samples in order of closeness	Aligned Amino Acids	Number of Amino Acids
11_	--SPCERVIFPT-AKYQILGKKFP-RTIKAPIIGAWSLPACLGHG-SNLAYL-GKILK-	52
15_	--SPCEHLNFPLRAQVSILGKKFP-STIKGSIFGAWSLPACFVHG-SILLTS-GKS---	51
10_	--SPCERRHFPLRAKYQILGKKFP-G-HRIVIFGAWSLPACFGPWLSILLYSPGKSFK-	54
2_	--SPCG-IHFPLRAKYQILGKQFP-STLKIVILGLG--PCRMRWPWLSLAYL-------	46
5_	---SLR-AYFPQ--QYQS-WKNSR-H-RPSWGLV---LASML-WPVAKLAYL-GNPK--	43
12_	---PFG-LIFLS--SINS-WKNSR-H-RASLGLG---LASML-WQWYNLAYL-GNPT--	43
16_	---HLQ-RYFLQ--AVSNSWKNSR-H-RPSLGLG---PCRMR--SWLNLAYL-GEI---	42
27_	---HLRGLIFPQ--QYQSRGRISR-H-RPSLGLG---PCRML--QWLNLAYL-G-----	41
22_	---PFS--AFPS--VSIKFWKNSR-H-RSSLGLG---PCLML-CQWLNLAYL-GKPLK-	44
31_	--PSAR--YFQS--ASXSSWKNSR-H-RPPLGLG---LAACF--GNGHLAYL-GETIK-	44
25_	--PSLS---FQR--PVSNSWKNFQ-T-LKIVIFGAWSLPACF-GQWLNLAYL-GENPK-	47
14_	---SLA--VYFP--QQYQFLEEFQ-T-LKIVIFGAWSLPACL-DDGSILLTS-GEIL--	46
28_	---HRA--AFSP--QQYQFVEEFQ-T-LKIVIFGAWSLPACF-G-GSILHTS-GEIP--	45
8_	-SPSAC--DFPT--ASIKFVEEFQ-T-LKIVIFGAWSLPACF-N-GSILLTL-GETPK-	48
21_	---PSA--HISI--PVSTIWKNFQ-T-LKIVIVGAWS------LPHALTMRS-SCFP--	41
32_	----SA--LISI--HASTIRGEFQ-T-LKIAIFGAWPC-RML-CQWTILLTS-GKLLK-	45
24_	---PCGGLNFPIQPRYRILGKNFQ-TIKEL-YRGLGPCQHAL--TMKQSC---------	43
29_	---PLRAFNFPLRGKYQIRGKEFPGQLKDSPSLGLGPCQHAL--GMAQSCP-RGKSFKI	53
9_	--SPCGAVIFPTASIN--SWQEFP-DIKDSRSLGLGPCQHAL--TMAQSCLPRGKS---	49
17_	----HLRRLISFQP----VSNSWK-NSRHRASLGLGPCLHAL--SMAQSCLPPGY----	44
20_	----SVG-AFPFR-----QYQSWK-NSRHRASLGLGPCQHAL--AMAQSCQPRGK----	42
26_	---PLRRLIFPYEPSIK-FVEEFPGLLKNSSIFGAWSLPACF--VNGQSCLPRGK----	49
30_	---PLQR--FSLQPSIK-FMEAISRLLKDSSSLGLGPASMPV--TMKQSCLPRGKSLK-	50
1_	--SPSA--VFSSA---SINRGRIP-DIKDSHLWG--LVLAACFGM-AQSCIPRENP---	45
6_	--SPSS--TFSLS---SINLGRIP-DIKDSHLWG--LVPARLLPV-APILLTW------	42
7_	--SPSAR-IFSQQ---YQIRGRIP-DIKDSHLWG--LVRAACLDN-ETILLTSGKI---	46
23_	---PFR--LIFLS---SINRGRIP-DIKDSHLWG--LVLASMLCP-NNLAYLGKSLK--	45
13_	HLASINF-PYSQI---SNSWKQFP-DIKDSHLWG--LVLASMPCQWLSLAIPRGNPKTC	52
19_	---HLAAPKFPLS--QYQMRGRIS---RHRSIFGAWSLPACF--VYGSILLT-SEI---	45
18_	---HLAS-AFPFQ--PVQFLEEFPAPLKIVDLWG--LVLACM--HRNNLAYL-GKI---	45
3_	---SLQR-YFPS---AVQFVEEFQ--TLKIASWG-LVLPHAL----TTIMLPREPL---	42

Figure 4.ClustalW (1.83) multiple sequence alignment. The amino acids sequences were aligned using clustalW software. The sequences are aligned with each other, with the query sequence (the sequence with all the amino acids found in other samples) at the top and subsequent sequences below. Gaps (spaces formed when two clusters are aligned by an extension of the pairwise alignment) are represented by "-" symbol. The running total numbers of amino acids are shown on the right; the aligned amino acids are shown at the centre while the running samples are shown on the left.

3.5 Generation of phylogenetic tree

The phylogenetic tree was used to determine polymorphisms in the CCR5 gene as evidenced by the presence of six different clusters (A-F; Figure 5). Based on the phylogenetic tree generated, it was seen that there were six main phylogenetic groups of the CCR5 gene (Figure 5) suggesting that the gene is highly polymorphic



Key:
Samples 1-32……Samples from the 8 provinces of Kenya.
A-F……CCR5 gene clusters

Figure 5. Phylogenetic tree of CCR5 gene sequences from the 8 provinces of Kenya based on 4 sequenced samples per province. It was generated by clustalW software to show how closely related the aligned sequences were.

4. Discussion

4.1 CCR5-Δ 32 Gene Mutation Frequency in Kenya and Possible Explanation of Disparity of HIV Prevalence in the Different Parts of the Country

This study involved the use of 200 samples from the eight provinces of Kenya that is 25 per province. The study aimed at explaining the differences in HIV prevalence in different parts of the country by presence and distribution of CCR5-32 bp polymorphisms. The results showed that there is no evidence of CCR5-Δ 32 bp mutation in the Kenyan populations. This is because, from the gel electrophoresis results, all the bands observed were estimated to have a molecular weight of 179 bp as opposed to 147 bp which would have been the case if CCR5-Δ 32 bp mutations were present. Although this study analyzed only 200 samples in the country, the data suggested that CCR5-Δ 32 bp deletions do not necessarily play any important role in the differences in the HIV prevalence in different regions of the country where Nyanza and Nairobi provinces has the highest HIV prevalence while North Eastern has the lowest. Other factors therefore, could be the course of this disparity that ranged from 1% -4% in 2003 and from 1%-30% in 2004 (Central Bureau of Statistics, Kenya Demographic and Health Survey, 2004: Populations Projections, CBS and NASCOP, 2005).

Although CCR5-Δ 32 bp deletions do not necessarily play an important role in the differences in HIV prevalence in different regions of the country, its absence could possibly be used to explain the severity of the disease as compared to populations in European countries where the allele is present. This is because Δ 32/Δ 32 homozygosity are protected against acquisition of HIV-1 by the mucosal route (Littman and Unutmaz, 1997) which is the main route of infection in Kenya and the world at large while heterozygous progress slowly to AIDS. This study confirmed the findings by other studies that CCR5-Δ 32 mutation is rare in African population

(Karam *et al*., 2004). The studies were done independently on non-Caucasians and revealed that this mutation is rare in non-Caucasian populations. A north to south gradient in the delta 32 allele frequency has been reported across Europe, with the highest allele frequencies in the Finnish and other populations living around the Baltic Sea (10%–20% heterozygous; 1% homozygous), and the lowest in Sardinia and Greece, where the frequency drops to almost zero (Martinson *et al*., 1997; Libert *et al.*, 1998; Magieronska *et al.*, 1998). The mutation is also seen at very low frequencies in populations from Saudi Arabia, Syrian Arab Republic, Islamic Republic of Iran, Tunisia, Morocco, Cyprus (Greek), India, Pakistan and Asia. It is rare in native populations from sub-Saharan Africa and Oceania (Lucotte, 2001).

Lack of the CCR5-Δ 32 mutation in the Kenyan population is consistent with the location of the origin of the mutation in northern Europe. In addition, frequencies of the deletion gradually decrease as the distance from Europe becomes greater and it is rare in Asia, the Far East, Oceania and South Africa (Karam *et al*., 2004). In addition to the gradient seen in Europe, a gradient outside Europe also exists for the mutation across the Middle East region and into Asia, the Far East and Oceania and across Europe into Africa. This is in accordance with a single point of origin for the mutation located in northern Europe, where the highest frequencies for the deletion have been reported (Karam *et al*., 2004).

The differences in prevalence of HIV in different parts of the country may be due to cultural practices, religious backgrounds and socio economic status and other factors, but not due to CCR5-Δ 32 mutations. For example, Nyanza province has the highest HIV/AIDS prevalence possibly due to cultural practices such as wife inheritance and widow cleansing. In addition to harmful cultural practices, the lower status of women, the social stigma surrounding HIV/AIDS and extreme poverty are contributing factors to the high HIV/AIDS prevalence rate in the province (Zulu, 1996; Bracher *et al*., 2002; Luke, 2002).The fact that the Luo as a community do not practice circumcision is also considered a factor in the high incidence of HIV/AIDS in Nyanza (Auvert *et al.*, 2001). Nyanza is also a major overland trade route. The main trucks stop on the interstate highway feeding Uganda, Rwanda, Burundi and Congo are in Kisumu city (Nyanza province) and Busia town in Western migration. This causes the movement of large numbers of young men and increasing women to cities, to large commercial farms and these contribute to high prevalence rate in this area (Zulu, 1996; Bracher *et al*., 2002; Luke, 2002).

Malaria too, which is endemic in this area might influence HIV transmission as it is associated with transient increase in semen HIV viral loads and thus could increase the susceptibility of the population to the HIV epidemic (Hoftman *et al.*, 1999). High HIV prevalence in Nairobi province may be as a result of its high population and the fact that it is the business centre as compared to other provinces. Islamic religion in North Eastern, which condemns infidelity, nomadic practices and low population, may have contributed to low HIV/AIDS prevalence in the area (Hoftman *et al.*, 1999). Many other demographic and social characteristics are associated with HIV infection. The wealthiest quintile of the population has the highest prevalence, nearly 10%, while prevalence among the poorest is less than 4%. Men who sleep away from home more than 5 days in a month have 3 times the prevalence of those who never sleep away 5 or fewer nights. Women who are widowed /divorced/separated also have high rates of HIV infection.

Though this study showed that the CCR5-Δ 32 mutations is absent in the Kenyan population, it is conceivable that other mutations in the CCR5 gene which disrupt its function as a co-receptor may account for some instances of this natural resistance to HIV-1 in Kenyan population like the prostitutes in Nairobi that were found to be resistance to HIV (Rupert *et al.*, 2000). A preliminary genetic survey of several major ethnic groups in the world where CCR5-Δ 32 mutations are rare has found several other sequence variations in the CCR5 locus, including a frame-shift mutation in the last transmembrane region that knocks out the last 54 amino acids (Smith *et al.*, 1997; Kostrikis *et al.*, 1998). This polymorphism, designated "del893C," is present at an allelic frequency of 0.04 in the Chinese and Japanese populations and could be severe enough to knock out the co-receptor function of the molecule. Therefore, exposed uninfected individuals in the Kenyan populations should be screened for this polymorphism, and proper i*n vitro* studies for co-receptor function performed with this mutant allele.

Other genetically host factors affecting HIV susceptibility and disease progression include a CCR5 A/A to G/G promoter polymorphism (McDermotte *et al*., 1998), a CCR2 point mutation (Smith *et al.*, 1997; Kostrikis *et al.*, 1998) and a mutation in the CXCR4 ligand (Winkler *et al*., 1998). The CCR2b mutation designated as CCR2641 is found in linkage with at least one CCR5 promoter polymorphism (Martinson *et al.*, 1997) and is prevalent in populations where CCR5-Δ 32 is nonexistent. Recently, a polymorphism consisting of a conserved valine to isoleucine change in the first transmembrane segment of CCR2b was discovered to be associated with delayed progression (two to four years) to AIDS (Martinson *et al.*, 1997). Other genes which influence disease progression include the major histocompatibility complex (MHC; human leukocyte antigen (HLA) class I and II) genes; these influence host immune responses. For example, persons with full heterozygosity at HLA class I loci have a slower rate of disease progression; this is presumably because the greater diversity of available class I molecules allows these individuals to respond to an increased number of viral antigenic epitopes (Kaul *et al*.,

2000). For instance, a small group of sex workers in Nairobi, Kenya, has remained uninfected by HIV-1 despite intense exposure and are defined as resistant to HIV-1 infection (Fowle *et al*., 1996). HIV-1 resistance in this cohort was not due to altered cellular susceptibility to HIV-1 or to known chemokine-receptor polymorphisms (Fowle *et al.*, 1998). This has been associated with certain HLA class I and II alleles (MacDonald *et al*., 2000), $CD8^+$ responses to HIV-1 CTL epitopes (Rowland *et al.*, 1998); Kaul *et al.*, 2000), T-helper responses (Kaul *et al*., 2000), and HIV-1–specific IgA (Kaul *et al*., 2000).

5.2 Polymorphism of CCR5 Gene in the Kenyan population

After analyzing all the PCR products by gel electrophoresis where it was revealed that CCR5-Δ 32 bp mutation do not exist in the Kenyan population, DNA sequencing was done to determine the genotypes of the PCR products that were amplified. The sequencing confirmed that the amplified PCR products were genotypes of the CCR5 gene. The nucleotide sequences of the CCR5 gene generated from the 32 samples that were sequenced were translated to amino acids and analysed using the clustalW software (Higgins *et al*., 1994). From the analysis, it was seen that there were amino acid sequence differences among them showing that the CCR5 gene is not highly conserved in the Kenyan population. Such an analysis has not been done before. In other related studies, scientists have only evaluated for the presence or absence of the Δ 32 base pair mutations but not for amino acid conservation.

To determine the phylogenetic similarities or differences among these sequences, a phylogenetic tree was constructed using the Treeview (Page, 1996) phylogenetic tree construction software. From the analysis, 6 different distinct clusters resulted indicating that there were 6 different phylogenetic groups of the CCR5 gene in the population studied. This was an indication that the CCR5 gene is highly polymorphic (Fig. 4.5). This is the first time that such a sequence phylogenetic analysis has been done indicating the phylogenetic differences of the CCR5 gene.The two findings mentioned above may have implications in the efficiency of the CCR5 co-receptor usage in the Kenyan population. It is important that studies be conducted to determine whether the sequence polymorphisms of the CCR5 gene may have implications on the efficiency of HIV transmission in the Kenyan population.

5. Conclusion

The study concluded that CCR5-Δ 32 bp mutations do not exist in the sampled population from Kenya. All the samples analyzed had a molecular weight of 179 bp as opposed to 147 bp indicating that there is no Δ 32 mutation.

The study also concluded that CCR5-Δ 32 bp deletions do not seem to play any important role in the differences in the HIV prevalence in different regions of the country where Nyanza and Nairobi provinces has the highest HIV prevalence while North Eastern has the lowest. Other factors like cultural practices, religious backgrounds and socio economic status therefore, could be the course of this disparity in HIV prevalence in different parts of Kenya.

It was also found that CCR5 gene is not conserved in the sampled population from Kenya. It also revealed that some regions of the gene in all the samples had same order of the bases suggesting that some regions are conserved while others are not and that CCR5-Δ 32 gene is highly polymorphic in the sampled population from Kenya.

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