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Full Length Research Paper

Sequence variation and co-receptor tropism prediction of V3 loop of HIV-1 from Kelantan, Malaysia

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V3 loop of human HIV-1 gp120 is a major determinant for T cell and macrophage tropism by determining the co-receptor used by the virus in the cell entry. Mutations in this motif could affect the syncytium formation, virus infectivity, neutralization, replication efficiency and host cell tropism. This study was aimed to determine the sequence variation of gp120 V3 loop for the prediction of co-receptor used by HIV-1 from Kelantan, host cell tropism and prognosis of disease progression. The V3 loop tip motif sequences of 30 HIV-1 from Kelantan, Malaysia were amplified, sequenced, analyzed and predicted by their co-receptor using a co-receptor usage prediction without sequence alignments: an application of string kernels (ds)Kernel bio-informatics software. The sequences were also subjected to the phylogenetic analysis for subtyping of the virus. The result revealed that among the 30 HIV-1 from Kelantan tested, 29 viruses were predicted to use CCR5 co-receptor and one virus utilised CXCR4. Phylogenetic result showed that 29 HIV-1 from Kelantan were of the unique recombinant form (URF) derived from CRF33_01B and only one virus was clustered with CRF53_01B of Malaysian HIV-1. Hence, the variation of the V3 loop sequences could be used to predict the co-receptor used by the virus for the prognosis of disease progression of the HIV-1 patients and subtyping of the virus.

Key words: Human immunodeficiency virus type 1 (HIV-1), Kelantan, Malaysia, V3 loop, sequence variation, HIV-1 subtyping.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) establishes its infection in the host by infecting T and macrophages of CD+cells (Alkhatib et al., 1996). It enters the cells by binding its gp120 envelope to the CD4 receptor and co-receptor proteins: CCR5 and CXCR4 (Dragic et al., 1996; Cormier and Dragic, 2002; Boisvert et al., 2008). The hypervariable region 3 (V3) of the HIV-1 gp120 is shown to have an important role in virus infectivity (Stamatatos and Cheng-Mayer, 1993; Morris et al., 1994), neutralization (Hwang et al., 1992), replication efficiency (Ivanoff et al., 1991) and host cell tropism (Hwang et al., 1991; Cann et al., 1992; DeJong et al., 1992; Sundaravaradan et al., 2007). Virus tropism is determined by the co-receptor used by the virus, either CCR5 (R5), CXCR4 (X4) or both (R5X4 virus) (Hoffman et al., 2002; Sundaravaradan et al., 2007; Dybowski et al., 2010). It is also associated with the ability of the virus to induce syncitia formation in macrophages (M-tropic virus) and T-lymphocytes (T-tropic virus) (DeJong et al.,

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1992). The M-tropic virus is classified as a non-syncytium-inducing (NSI) virus that uses CCR5 as a co-receptor and predominates in the asymptomatic phase. The syncytium-inducing (SI) T-tropic variant uses CXCR4 as a co-receptor, presents in the advance stage of HIV infection and progresses faster to Acquired Immunodeficiency Syndrome (AIDS) (Connor et al., 1997). Therefore, an accurate prediction of the co-receptor used by the virus is important as it allows for prognosis of disease progression and selection of effective antiretroviral (ARV) drugs.

Current standard method for determining the co-receptor tropism is based on cell assays (Trouplin et al., 2001; Whitcomb et al., 2007). However, this assay can only be carried out in the laboratories with biosafety level III (BSL III) facilities and long turn-around time (4-6 weeks) have impelled the development of alternative genotypic methods. Due to the association between V3 loop sequences with viral receptor usage, a simpler genotypic assay based on a combination of V3 sequencing with bioinformatics tools such as Geno2pheno, Web PSSM and SVM have been developed and widely used (Jensen and Wout, 2003; Poveda et al., 2007; Sing et al., 2007; Boisvert et al., 2008; Sierra et al., 2011). Although the overall reliability of sequence-based methods for co-receptor prediction is still limited (Lengauer et al., 2007; Kuritzkes, 2011), these methods are well accepted as a way to define the HIV-1 cell tropism for monitoring the disease progression of HIV patients (Vandekerckhove et al., 2011). Therefore, this study was aimed to explore the variability of tip motif in the V3 loop of HIV-1 from Kelantan and to correlate it with virus tropism based on the co-receptor usage of the virus using (ds)Kennel (http://genome.ulaval.ca/hiv-dskernel/) bio-informatics software. This is very useful for the prognosis of disease progression of HIV patients from Kelantan, Malaysia and for personalized selection of effective drug therapy. The sequences were further subjected to the phylogenetic tree for subtyping of HIV-1 viruses from Kelantan, Malaysia.

MATERIALS AND METHODS

HIV-1 samples

Thirty confirmed HIV-1 plasma samples were collected from Pengkalan Chepa prison and Hospital Raja Perempuan Zainab II, Kelantan, Malaysia with an IRB approval from Universiti Sains Malaysia (USM) and Ministry of Health (MOH), Malaysia. All the samples were from the anti-retroviral (ARV) treatment-naive patients. Viral load and CD4 counts of the samples were previously determined as shown in Table 1.

V3 loop sequencing and genotype prediction

V3 loop of gp 120 HIV-1 envelope gene was amplified using AccessQuick RT-PCR (Promega, USA). Nucleic acid was extracted using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Designed primers: HIV $ENVN_2F$ (5' AGC CAG TGG TAT CAA CTC A 3') and HIV $ENVN_2R$ (5' CAA TAG AAA AAT TCC CCT C 3') were used for the amplification of V3 loop sequences. Sequencing of the PCR products was performed using ABI Prism 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) in triplicates. Sequences were analyzed using CromasPro software (Technelysium Pty Ltd), translated to amino acid sequences using Generunner program (Hasting Software, Inc.). Genotypic prediction of co-receptor tropism was based on co-receptor usage prediction without sequence alignments: an application of string kernels bio-informatics software (http://genome.ulaval.ca/hiv-dskernel/) that is built with Los Alamos National Laboratory HIV Databases sequences (Boisvert et al., 2008). V3 loop sequence of SR-4_X4/X4 (AF333214) and CNXJ1081_R5 (KC807945) were used as R4 and R5 reference strains, which respectively have the GPGR and GPGQ motifs at the V3 loop crown (amino acid position 15 to 18).

HIV-1 subtyping

HIV-1 subtyping was determined by phylogenetic analysis of V3 loop sequences using aligned sequences from the ClustalX program (NCBI) and Neighbour-joining (NJ) (Saitou and Nei, 1987) constructed phylogenetic tree. HIV-1 from Malaysia (06MMYKLD46_EF495062, 04MYKL005.1_ EU785955, 09MYSB023_JX390976 and10MYKJ079_JX390611) and Thailand (CM243_ HIVTN2432, CM240_ HIVU54771, 93TH253_U51189 and 02TH.OUR1331_AF529572) were used as reference subtypes. CNHN24_AY860947.1 and DU123_ AF544007.1 were used as out group viruses.

RESULTS

Phylogenetic analysis

Based on the phylogenetic tree data, 29 HIV-1 from Kelantan were clustered in a unique recombinant form (URF) CRF01_AE/B derived from CRF53_01B of Malaysian (04MYKL005.1) HIV-1. One virus (HR02) was clustered with 10MYKJ079 of the CRF53_01B from Malaysia (Figure 1).

Prediction of co-receptor usage by the viruses

An alignment of the V3 loop sequences of 30 HIV-1 showed that all the viruses have GPGQ motif at the V3 loop crown which was similar to R5 reference strain: CNXJ1081 (Figure 2). However, the co-receptor usage prediction result shows that 29 of these viruses were predicted to use CCR5 as a co-receptor while one virus

Table 1. List of HIV-1 samples used in this study.

S/N	Samples	Viral load copies/ml	CD4 count/mm^3	Risk group
1	HR02	216,495	214	IVDU
2	HR06	79,995	269	Sex
3	HR07	57,000	297	Sex
4	HR08	67,564	287	Sex
5	HR09	189,723	447	Sex
6	HR10	1,761	492	Sex
7	HR13	7,574	407	IVDU
8	HR14	18,410	358	Sex
9	HR15	392,961	222	Sex
10	HY01	134,978	159	IVDU
11	HY02	100,280	120	IVDU
12	HY03	55,122	307	IVDU
13	HY04	3,420	199	IVDU
14	HY05	15,324	209	IVDU
15	HY08	281,278	345	IVDU
16	HY32	63,221	168	Sex
17	HY33	23,186	332	IVDU
18	HY36	15,876	234	IVDU
19	HY37	97,723	434	IVDU
20	HY38	80,600	520	IVDU
21	HY40	28,348	478	Sex
22	HY41	102,162	412	IVDU
23	HY44	114,141	367	IVDU
24	HY45	43,049	224	IVDU
25	HY49	115,598	380	IVDU
26	HY50	78,224	91	IVDU
27	HY51	32,414	446	IVDU
28	HY52	44,651	263	IVDU
29	HY53	17,846	314	IVDU
30	HY56	35,388	373	IVDU

*IVDU-Intravenous drug user.

(HY49) used CXCR4 as a co-receptor (Table 2).

DISCUSSION

Due to the simplicity of genotypic assay for the determination of host cell tropism, it is recommended to be used in the clinical practice for prognosis of disease progression and selection of effective antiretroviral (ARV) drugs. In this study, the prediction of co-receptor used by the HIV-1 from Kelantan was carried out using (ds)Kennel bio-informatics software. This software predicts the co-receptor usage of the HIV-1 samples based on the translated amino acid sequences, built with Los Alamos National Laboratory HIV Databases sequences. The prediction accuracies for R5 and R4 viruses are 96.35 and 94.80% respectively (Boisvert et al., 2008). Based on the values, the results predicted are reliable but are recommended only for research purposes (Boisvert et al., 2008).

Most of the subtype C CXCR4-using viruses have been shown to have a conserved GPGQ sequence at the V3 tip motif, while CXCR4-using viruses have R substitutions (GPGR) in this motif (Coetzler et al., 2006). The results of this study reveal that 29 (97%) out of 30 viruses were predicted to use CCR5 co-receptor and only one virus (HY49) used CXCR4 co-receptor. All these viruses including the predicted R4 virus (HY49) were shown to have a GPGQ motif at the V3 crown, which is similar to the R5 reference strain. However the predicted R4 virus was shown to have lysine (K), glutamic acid (E) and leucine (L) amino acid substitutions at the 22, 25 and 30th position of V3 amino acid sequence respectively (Figure 2), that might contribute to the CXCR4 co-receptor usage of this virus. This result is further

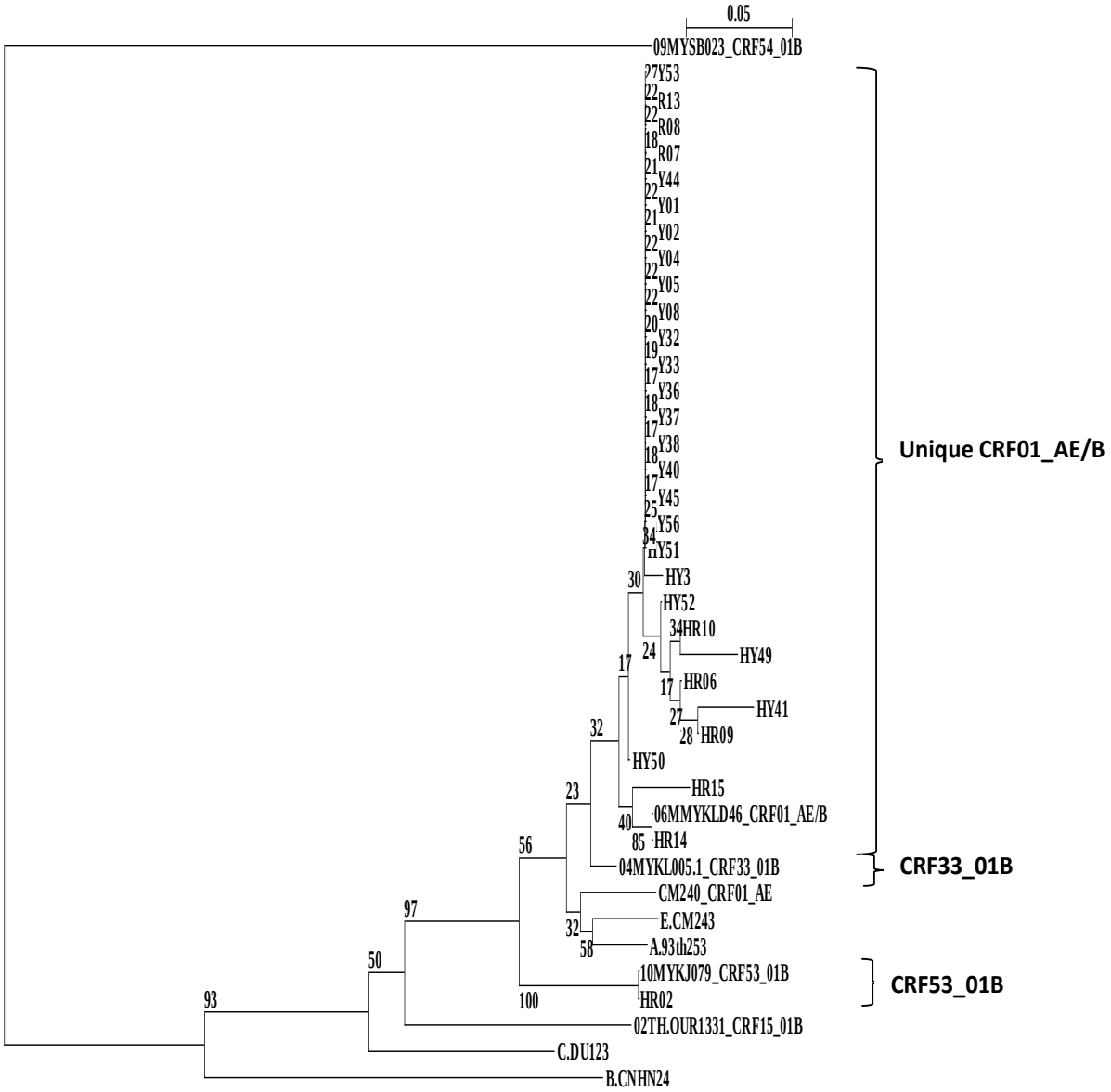

Figure 1. The phylogenetic tree showing the V3 loop nucleotide sequence relationships of 30 HIV-1 viruses from Kelantan to other strains from the NCBI GenBank including those from Malaysia (06MMYKLD46, 04MYKL005.1, 09MYSB023 and 10MYKJ079) and Thailand (CM243, CM240, 93th253 and 02TH.OUR1331).
CNHN24 and DU123 were used as outgroup viruses. The sequences were aligned using ClustalX (NCBI) and the tree was constructed by Neighbor-joining (NJ) method. Numbers adjacent to each branch represent the percentage bootstrap calculated for 100 replicates. The scale bar represents 5% of genetic distance (0.05 substitutions per site).

supported by Coetzer et al. (2006), where only 75% of the R4 viruses of subtype B have the GPGR motif at their V3 crown.

It has been shown that the risk of X4 virus emergence is related to the drop of CD4+ T-cell counts below the critical threshold of 200 cells/mm^3 (Tomasini-Grotto et al., 2010). However, in this study, the predicted R4 tropic patient (HY49) has CD4 count more than 200 cells/mm^3. It can be assumed that the infection is still in the early stage of switching to AIDS stage, where the patient's immune system is still able to control the virus but will be decreased as the infection progresses. There were 2 patients (HY01, HY02) that have the CD4 count less than 200 cells/mm^3 with viral load more than 100,000 copies/ml (suspected AIDS progression) but were predicted to use CCR5 co-receptor. Similar finding was also observed by Coetzer et al. (2011), where the co-receptor switching was very rare in subtype C viruses,

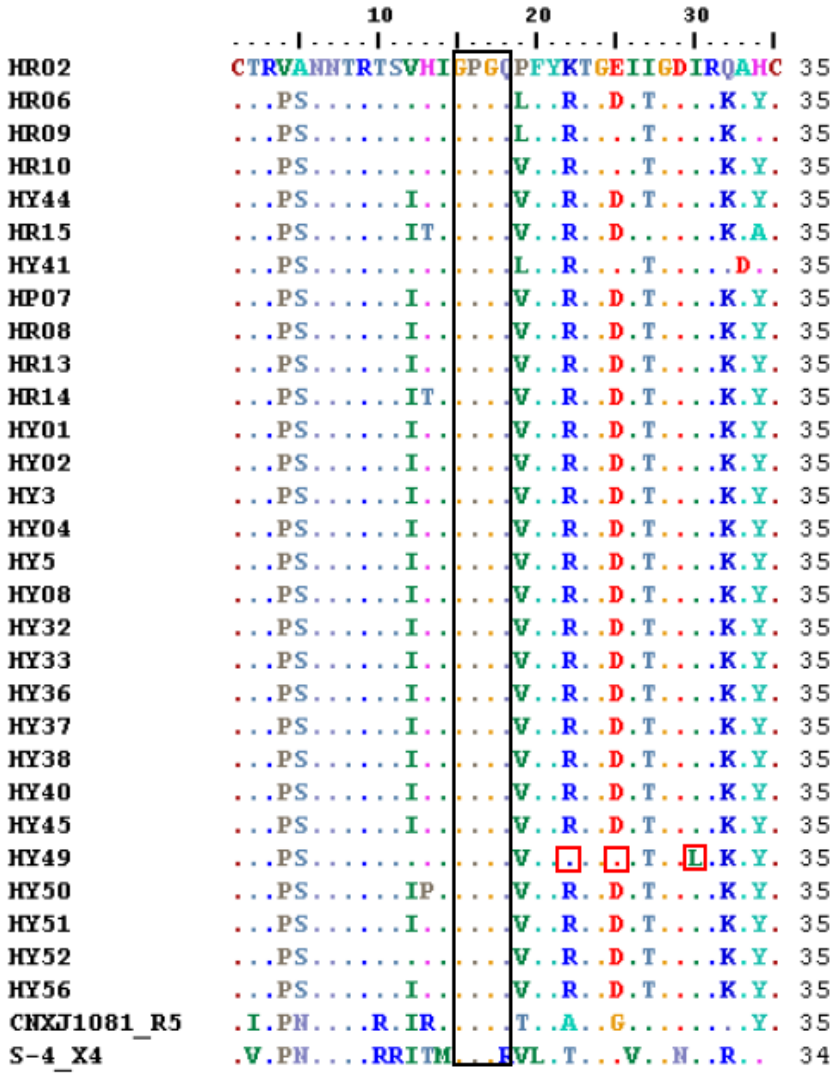

Figure 2. Amino acid sequence alignment of 30 HIV-1 samples from Kelantan, Malaysia using Bioedit program. SR-4_X4/X4 and CNXJ1081 were used as a R5 and R4 reference strains respectively. The black box indicates the position and sequence of tip motif of V3 crown and red boxes indicate the critical amino acid mutations that might contribute to the CXCR4 co-receptor usage of HY49.

including in the AIDS stage, suggesting that other factors such as host immune or virological constraints may be involved in limiting the development of these viruses to the R4 tropic viruses. For further confirmation of this tropism, phenotypic assays based on recombinant viruses need to be carried out and information on the clinical status of the patient should also be included for a better co-receptor prediction using genotypic bio-informatics tools (Low et al., 2007; Sing et al., 2007).

Phylogenetic tree result showed that 29 HIV-1 from Kelantan were identified as unique recombinant CRF01_AE/B that was derived from CRF53_01B of Malaysian HIV-1(04MYKL005.1). One virus (HR02) was clustered with 10MYKJ079 of the CRF53_01B from Malaysia. These subtypes emerged due to the co-circulation and dual infection of CRF01_AE and B subtype in HIV-1 patient (Lau et al., 2010). Multiple unique recombinant forms (URFs) derived from CRF33_01B have been reported to disseminate widely among various HIV-1 risk populations in Kuala Lumpur, Malaysia (Chow et al., 2014), indicating an extensive recombination occurrence in Malaysia. However full-length genome sequencing of these URFs has to be performed to confirm these viruses as a novel circulating recombinant form (CRF) virus. As a conclusion, of the 30 HIV-1 from Kelantan analysed, only one virus (HY49)

Table 2. V3 tip motif sequence and predicted co-receptor used by HIV-1 from Kelantan, Malaysia using (ds)Kennel bio-informatics software.

S/N	Samples	V3 tip motif sequence	Predicted co-receptor usage
1	HR02	GPGQ	CCR5
2	HR06	GPGQ	CCR5
3	HR07	GPGQ	CCR5
4	HR08	GPGQ	CCR5
5	HR09	GPGQ	CCR5
6	HR10	GPGQ	CCR5
7	HR13	GPGQ	CCR5
8	HR14	GPGQ	CCR5
9	HR15	GPGQ	CCR5
10	HY01	GPGQ	CCR5
11	HY02	GPGQ	CCR5
12	HY03	GPGQ	CCR5
13	HY04	GPGQ	CCR5
14	HY05	GPGQ	CCR5
15	HY08	GPGQ	CCR5
16	HY32	GPGQ	CCR5
17	HY33	GPGQ	CCR5
18	HY36	GPGQ	CCR5
19	HY37	GPGQ	CCR5
20	HY38	GPGQ	CCR5
21	HY40	GPGQ	CCR5
22	HY41	GPGQ	CCR5
23	HY44	GPGQ	CCR5
24	HY45	GPGQ	CCR5
25	HY49	GPGQ	CXCR4
26	HY50	GPGQ	CCR5
27	HY51	GPGQ	CCR5
28	HY52	GPGQ	CCR5
29	HY53	GPGQ	CCR5
30	HY56	GPGQ	CCR5

utilized CXCR4 co-receptor and the other 29 viruses used CCR5 co-receptor with all of them displaying GPGQ motif at the V3 crown. Phylogenetic analysis result based on V3 loop sequence revealed that 29 of these viruses were of the unique CRF01_AE/B subtype derived from CRF33_01B and one virus (HR02) as a CRF53_01B recombinant subtype.

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