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Comparison of dynamics of HIV-1 coreceptor usage in a long-term antiretroviral treatment adolescent by genotypic and phenotypic assays

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ABSTRACT

Background: Based on coreceptor usage, HIV-1 variants can be classified as R5, X4, and dual/mixed viruses. Currently, the determination of HIV-1 coreceptor usage can be performed by both phenotypic and genotypic assays. Although, the accessibility, simple, and low cost makes those genotypic assays a more feasible alternative to phenotypic assays, but they are not always accurate. Here, we discussed the coreceptor usage obtained by both assays in HIV-infected patient who acquired HIV-1 CRF01_AE and received antiretroviral therapy for at least 10 years.

Objectives: To determine the HIV-1 coreceptor usage by both genotypic and phenotypic assays at different three times in long-term antiretroviral treatment adolescent.

Materials and methods: The remained RNA was collected at different three times to determine the HIV-1 coreceptor usage by both phenotypic and genotypic assays. Firstly, HIV-1 V3 region was amplified, sequenced, and then V3 amino acid sequences were used as templates for prediction of coreceptor usage by genotypic predictors. Secondly, the entire gp160 envelope fragment was amplified from the same remained RNA to produce env-pseudotyped virus. The viruses were tested for coreceptor usage using U373.R5 and U373.X4 cells, and viral entry was assessed with luciferase activity measurement.

Results: From all time points at which coreceptor usage was determined, the genotypic results showed that the coreceptor usage trend to be more X4 phenotype using genotypic predictors, but it contrasted with phenotypic result which only voted to R5 phenotype. Although, the genotypic results showed the evolution of V3 amino acid sequences but it still not sufficient for coreceptor changed when confirmed with phenotypic assay. The presence of positively charged amino acid in V3 sequences causes a high net charge which can lead to mis-prediction by genotypic predictors.

Conclusion: This finding suggested that the predictions are not always accurate; a false prediction of X4 variants may lead to unnecessarily precluding patients who could have benefited from receiving CCR5 inhibitors whereas a false prediction of R5 variants may lead to the reemergence of X4-strains under CCR5 inhibitors pressure. Thus, the utilization of genotypic predictors should be carefully considered.

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Introduction

This case was of a HIV-infected patient who acquired HIV-1 subtype CRF01_AE and received antiretroviral therapy for at least 10 years. He was born in 1998, and his HIV testing was positive in 2004. He has been virologically and immunologically followed up since then. This study was approved by ethical committees from the Faculty of Associated Medical Sciences, Chiang Mai University (Reference No. 008EXP/56). The HIV-1 viral loads and number of CD4 cell counts were observed during 2004-2015 as shown in Figure 1. The HIV-1 viral load was stable as 3-4 $\log_{10}$copies/mL between 2009 and 2011. In 2012, he had experienced a virological breakthrough when the viral load had reached a peak (6 $\log_{10}$copies/mL) together with a rapid fall in CD4 cell counts. However, in 2013, his viral load could only be detected as 1.53 $\log_{10}$copies/mL whereas the patients CD4 cell counts were increasing. His viral load increased to 4.34 $\log_{10}$copies/mL in 2015 whereas CD4 cell counts had slightly increased from 2014 to 2015.

This patient was promptly started on antiretroviral treatment that consisted of stavudine, lamivudine, and nevirapine during 2004-2009. During this time, the numbers of CD4 cell counts were only observed. In October 2009, he received HIV-1 drug resistance testing at our faculty for the first time. At this time, he had evidence of resistance to NRTIs and NNRTIs, but no evidence of resistance to PIs. Then, he started to receive indinavir and kaletra that consisted of lopinavir and ritonavir. In December 2010, he received HIV-1 drug resistance testing for the second time. The result showed no evidence of resistance to NRTIs and NNRTIs but he had evidence of resistance for PIs. However, he continued to receive the drug the same as previously until July 2012. At this time, he had experienced a virological breakthrough when the viral load had reached a peak (6 $\log_{10}$copies/mL) together with a rapid fall in CD4 cell counts (76 cells/mm^3). After testing the HIV-1 drug resistance, he showed no evidence of resistance for NRTIs and NNRTIs but still resistant to PIs. He has been continually followed up and treated with drug regimens consisting of lamivudine, stavudine, and kaletra.

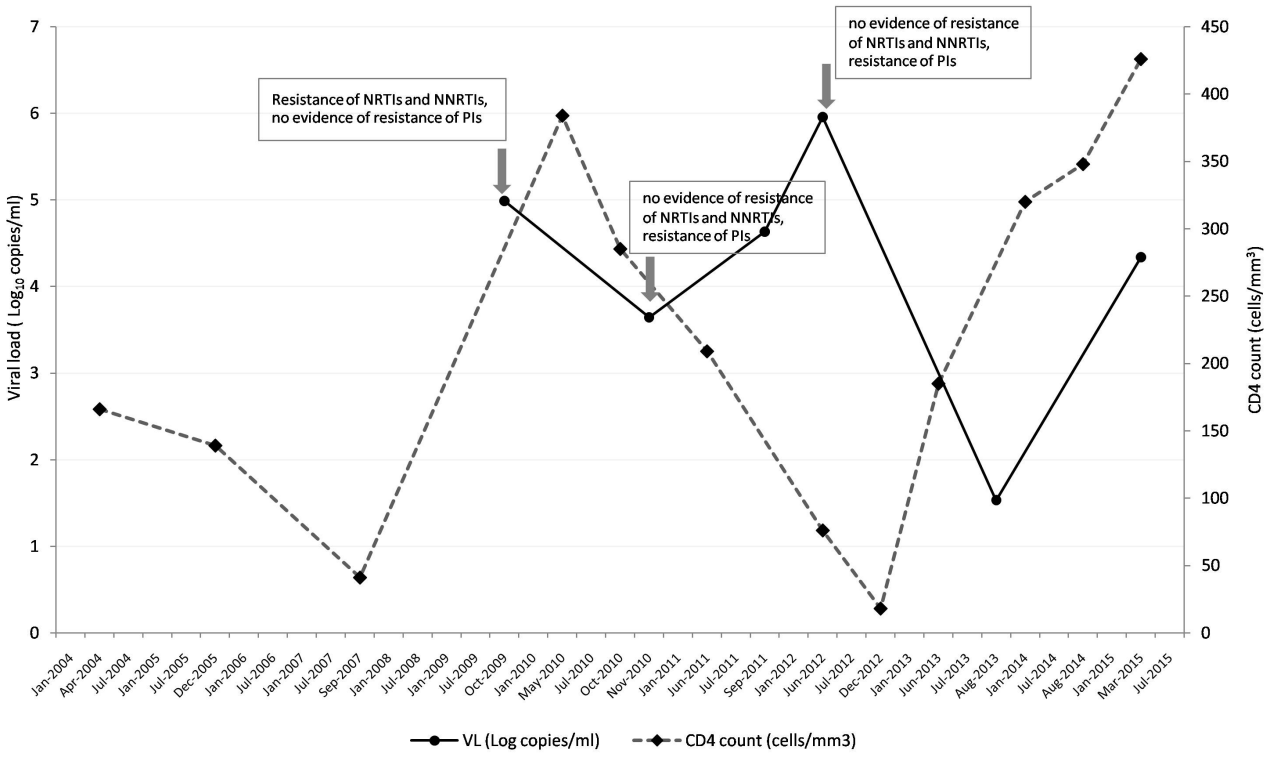

Figure 1. *Evolution of CD4 cell counts and HIV-1 RNA viral loads over 2004-2015. CD4 cell counts results are represented in diamonds (broken line) and HIV viral load in black circles (solid line). Vertical arrows at the top indicate the time points at which coreceptor usage was determined. The drug resistance results are presented in the box for each time point.*

Based on coreceptor usage, HIV-1 variants can be classified as R5, X4, and dual or mixed viruses.[1] X4 virus generally emerges in more advanced diseases and is associated with a sharp decline of CD4 T cells.[2-3] In this regard, we hypothesized that the coreceptor usage may be involved in the clinical pathogenesis during disease progression for this case. Then, the remaining RNA from HIV-1 drug resistant testing in three time points was collected to determine the HIV-1 coreceptor usage by both phenotypic and genotypic assays. The vertical arrows at the top of Figure 1 indicate the time points at which the coreceptor usage was determined.

Firstly, the V3 region was amplified from the remaining RNA and then this amplified product (~329 bp) was cloned using the pCR® 2.1 Vector TA cloning kit (Invitrogen, USA). Plasmid DNA containing V3 inserts (15-20 clones) were purified, and then directly sequenced using Big Dye Terminator v3.1 on an ABI3130 (Applied Biosystem). The V3 amino acid

sequences were then analyzed and used as templates for the prediction of coreceptor usage by the available genotypic predictors. Several algorithms have been presented for the prediction of coreceptor usage, including the 11/25 rule which predicts CXCR4 coreceptor usage if a positively charged amino acid (Arginine[R] or Lysine[K]) is presented at a position of 11 and/or 25, and if the overall net charge of V3 region is ≥5, a sample will be labeled as X4 according to the net charge rule.[4-5] Moreover, we also determine the coreceptor usage using bioinformatics tools (Geno2Pheno$_{[coreceptor]}$ and webPSSM) that are widely used and freely available on the website.[6-7] The V3 amino acid sequences and predicted coreceptor usage at three time points using different interpretation algorithms is shown in Table 1. At the first time point, the V3 region indicated that most of the clones (16/18 clones) had identical V3 amino acid sequences and most of genotypic predictors predicted as R5 phenotype. For the second time point, the V3 amino acid sequences were heterogeneous and several clones (12 clones) had an amino acid sequence equal to the first time point. Remarkably, five clones showed a high net charge because of the presence of positively charged amino acids (Arginine [R], Lysine [K]) in their sequences that lead to the prediction of X4. At the third time point, the result demonstrated that the V3 amino acid sequences were heterogeneous and different from previous points. These sequences were predicted as X4 phenotype by genotypic predictor, particularly by Geno2Pheno. Only one clone had an amino acid sequence as same as the prior. The genotypic results suggested that V3 amino acid sequences from those three time points were different and predicted that the coreceptor usage trend would be changed to an X4 phenotype. To confirm this finding, we repeated the determination of coreceptor usage by phenotypic assay.

Secondly, the phenotypic assay was performed to determine the coreceptor usage based on *env*-pseudotyped viruses. The entire gp160 envelope fragment was amplified from the same remaining RNA that was used to sequence in the V3 region as previously described. This envelope fragment (~2600 bp) was inserted into a PCI expression vector, transformed into the competent cells, and then the plasmid containing the HIV envelope fragment was extracted for pseudovirus production. This plasmid was co-transfected into the 293T cells with a full-length of the HIV gene without the envelope and luciferase was then used as a reporter. To determine the coreceptor usage, the pseudotyped viruses were inoculated in CCR5- and CXCR4-expressing cells. Viruses were then classified as R5, X4, or dual/mixed depending on their tropism. The phenotypic assay can only be performed at the first and third time points. At the first time point, the coreceptor usage showed as CCR5-using virus (mean Log Relative Light Unit (RLU) =5) and the coreceptor usage at the third time point was still served as CCR5-using virus (mean Log RLU=5.8). These phenotypic results suggested that the coreceptor changed was not found whereas the genotypic results were mostly predicted as X4 phenotype.

Table 1 V3 amino acid sequences and coreceptor usage at three time points determined by phenotypic assay and different interpretation algorithms.

Date of sample collection	V3 amino acid sequences	clone	Phenotype	Genotypic predictors						
				11/25 rule	net charge rule	G2p 1%	G2p 2.5%	G2p 5% and G2p 10%	G2p 15% and G2p 20%	PSSM
CM244	CTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYC									
Oct-2009	..........VH..........G......Q...	*bulk*	R5	R5	R5	R5	R5	R5	R5	R5
	..........VH..........G......Q...	16		R5	R5	R5	R5	R5	R5	R5
	.....T.....VH..........G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	..........VH..........G......R...	1		R5	X4	R5	R5	R5	X4	R5
Dec-2010	..........VH..........G......Q...	11	ND	R5	R5	R5	R5	R5	R5	R5
	.I..F.K.K...H....RM....K..G.T.K..H.	3		R5	X4	R5	R5	X4	X4	X4
	..........VH.....A.....G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	.I..F.K.K.N.H....RM....K..G.T.K..H.	1		R5	X4	R5	X4	X4	X4	X4
	..........VH..........S......Q...	1		R5	R5	R5	R5	R5	R5	R5
	...L.......VH..........G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	.I..F.K.K...H....RM....K..G.T.K.GH.	1		R5	X4	R5	R5	X4	X4	X4
	..........VH..........G......R...	1		R5	X4	R5	R5	R5	X4	R5
Jul-2012	.S.........TRM.................Q.H.	*bulk*	R5	R5	R5	R5	R5	X4	X4	R5
	.S.........TRM.................Q.H.	13		R5	R5	R5	R5	X4	X4	R5
	.S.........TR..................Q.H.	1		R5	R5	R5	R5	X4	X4	R5
	.SG........TRM........A........Q.H.	1		R5	R5	R5	R5	X4	X4	X4
	..........TRM.................Q.H.	1		R5	R5	R5	R5	X4	X4	R5
	..........VH..........G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	.....H.....VH..........G......Q...	1		R5	X4	R5	R5	R5	R5	R5
	..........GVHM.........G......Q...	1		R5	R5	R5	R5	R5	R5	R5

ND: Not done, G2p: Geno2pheno$_{[coreceptor]}$ at different false positive rate

Note: *The V3 sequence of HIV-1 CM244, used as the reference sequence, is presented at the top of column. Sequences of clones obtained at each time point are presented below with the number of clones sequenced. Changes in an amino acid are identified by a single-letter code and amino acids identical to the amino acid in the reference sequence are identified by dot. Coreceptor usage was determined using bulk phenotypic assays as well V3 sequence data interpreted with different algorithms are showed in the right.*

Discussion

Currently, the determination of HIV-1 coreceptor usage based on genotypic tools is available and widely used to predict the coreceptor usage from the V3 amino acid sequence. The accessibility, simplicity, and low cost makes those genotypic assays a more feasible alternative to phenotype assays. Several studies have determined the coreceptor usage based on V3 amino acid sequences instead of phenotype assays due to its superiority.[8-11] In fact, these tools have been developed based on the data of HIV-1 subtype B and C mainly, thus they are less accurate for non-B subtypes as previously reported.[12-14] Here, we discussed a patient who had evidence of a coreceptor usage trend showing more of an X4 phenotype using genotypic predictors, but it contrasted with phenotypic result, which only voted to R5 phenotype. Although, the genotypic results showed the evolution of V3 amino acid sequences which were quite different in the two years between the first and third time points, but it is still not sufficient for coreceptor changed when confirmed with phenotypic assay. Notably, genotypic assays typically predict the coreceptor usage as X4 if the V3 sequence contains more positively charged amino acids. The presence of these positively charged amino acids in V3 sequence causes a high net charge, which can lead to the prediction of X4 by genotypic tools. Furthermore, a previous study also reported that Geno2pheno$_{[coreceptor]}$ and WebPSSM overestimated CXCR4 usage for CRF01_AE and CRF02_AG subtype,[15] which were also found in this study. The discordant results between genotypic and phenotypic assays could be raised from the comparison of coreceptor usage predictions based on V3 amino acid sequence only to a phenotypic assay taking the whole envelope region. The other domains in gp120 V1, V2 as well as in gp41 region have also been reported to influence the coreceptor usage.[16-18] Thus the prediction based on V3 sequences only may impact the reliability of tools. The other reasons could account for discordant results between genotypic and phenotypic assay. Of note, the genotypic tools have been specifically developed based on subtype B and C mainly, thus using these tools for determining the coreceptor usage of non-B and non-C subtypes have not been turned specifically. Moreover, it may be important that when clonal sequencing is used for genotypic assay, all possible clones are genotyped, and a prediction is provided for each clone. In this phenomenon, genotypic tools would infer tropism for sequences that do not exist in the viral population, eventually leading to an overestimation of prediction, whereas the phenotypic assay only measures existing strains or dominant viral population.

This finding suggested that genotypic tools are not always accurate for predicting HIV-1 coreceptor usage and the phenotypic assay is still a reference method, which is the most accurate method for the determination of HIV coreceptor usage, thus the utilization of genotypic predictors should be carefully considered. A false prediction of X4 variants may lead to unnecessarily precluding patients who could have benefited from receiving CCR5 inhibitors whereas a false prediction of R5 variants may lead to the reemergence of X4-strains under CCR5 inhibitors pressure. Further studies need to improve the prediction tools when non-B subtypes are involved or new subtype-specific rules should be developed to accurately predict the coreceptor usage and avoid the exclusion of patients who need to access the coreceptor inhibitors.

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