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Full Length Research Paper

Impact of nucleotide polymorphisms at drug resistance sites on genetic barrier in human immunodeficiency virus type 1 subtype C resistance evolution

Benson Chuks Iweriebor[1]*, Tracy Masebe[1], Julius Nwobegahay[1], Jeffrey M. Mphahlele[2] and Pascal Obong Bessong[1]

[1]AIDS Virus Research Laboratory, Department of Microbiology, University of Venda, South Africa.
[2]HIV and Hepatitis Research Unit, Department of Virology, University of Limpopo, Medunsa Campus, South Africa.

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Genetic diversity is the hallmark of human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS). This diversity has resulted in a spectrum of different subtypes within the group M viruses that is responsible for the AIDS pandemic. Nucleotide substitutions and polymorphisms at codons known to confer drug resistance in subtype B viruses were compared with similar substitutions in subtype C viruses. Genetic barrier was determined on viruses isolated from drug naive patients infected with subtype C viruses. We found a reduced genetic barrier in subtype C viruses at codon V106M (GTA to ATG) and an increased barrier at codon L210W (TTA/CTG/CTA to TGG) when compared to subtype B consensus. The highest genetic barrier in subtype C viruses is found at codon Q151M where two transversions or each one of transition and transversion are needed for the resistance evolution.

Key words: Human immunodeficiency virus (HIV), drug resistance, genetic barrier, non-B subtypes.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) is characterized by extensive genetic diversity occasioned by several factors which includes the rapid turnover of HIV-1 *in vivo* (Ho et al., 1995), host selective immune pressure (Michael., 1999), infidelity of the reverse transcriptase; the enzyme responsible for the synthesis of viral DNA from RNA, due to its lack of proof reading capability and the propensity of the virus to undergo recombination events during replication (Temin, 1993). As a result of this variability, HIV-1 variants are classified into four major phylogenetic groups: groups M, O, N (Gurtler et al., 1994; Ayouba et al., 2000; Simon et al., 2003) and the newly identified group P (Plantier, 2009). Group M which is responsible for the majority of infections in the HIV-1 pandemic can be further subdivided into nine recognized phylogenetic subtypes A to D, F to H, J and K, all of which are equidistant from one another. Within this same group, the average inter-clade genetic variability is about 15% for the gag gene and above 25% for the envelope gene and about 10% for the pol gene (Janssens et al., 1994; Kostriks et al., 1995; Leitner et al., 1995). Also within a subtype, it is possible to identify sub-subtypes which are groups of viral strains forming genetically related clades that are more closely related phylogenetically to each other that with other subtypes. This is commonly found in subtypes A and F whose members are currently classified into A1 to A5 (Niama et al., 2009) and F1 and F2 sub-subtypes, respectively (Gao et al., 2001). Coupled with these subtypes, are numerous circulating recombinant forms (CRFs) and unique recombinant forms (URFs) which are thought to have originated from individuals who are either co-infected or super-infected. Currently, there are 49 CRFs and numerous URFs in the Los Alamos database (www.hiv.laln.gov) that are driving the epidemic in different geographical regions of the world.

The diversity of the pol gene is also due to the factors that characterize diversity in other gene regions. As a result of the degeneracy of the genetic code, more than

*Corresponding author. E-mail: benvida2004@yahoo.com. Tel: +27 15 962 8301. Fax: +27 15 962 4749.

one codon can code for a particular amino acid resulting in nucleotide polymorphisms in the pol gene of the different subtype. For example, subtype D viruses are characterized by having GAT codon at position D67, whereas GAC is predominant in other subtypes. At position K70 in the RT, AAA codon is present in subtype B viruses, while AAG is generally encoding K in subtypes C and D viruses. Also, at codon K219, AAA in subtype B is replaced by AAG in most subtypes C viruses. Similarly, AAC is the codon that predominates in subtype B viruses at position T215, whereas ACT encodes T215 in most A and all recombinant A/E viruses. Also, V106 is encoded by GTG in majority of subtype C viruses and about half of subtype D viruses. At codon V179, GTG predominates in subtype A/G and G viruses with GTC in subtype C viruses. Similarly, a TAC codon that encodes Y181 is characteristic of subtype G viruses and in the majority of the A/G recombinants, while the TAT codon is predominant in subtypes B, C and A/E viruses, and CCC is predominantly found in subtype C at position P225. These polymorphisms if conferring drug resistance can be selected by the drug-selective pressure and dramatically influence therapeutic outcome (Buonaguro et al., 2007). On the other hand, these subtype specific polymorphisms many not confer drug-resistance but may change the genetic barrier which is the number of viral mutations needed to overcome the drug selective pressure.

The frequency and pattern among HIV-1 subtypes of polymorphism inducing resistance or resulting to a faster emergence of drug resistance once under drug pressure has been evaluated extensively on subtype B (Turner et al., 2004; Pillay et al., 2002). This information is scarce in non-B subtypes which predominate in regions where ARV is becoming more available and yet have a heavy burden of the disease. Although most current anti HIV-1 drugs were designed for use against subtype B variant which is responsible for the epidemic in the western world, there is no compelling evidence to suggest that they are any less effective against other subtypes. Even though the drugs are effective against all the subtypes, it is not known whether resistant development will be different in non-B viruses than in B subtype when the virus is under drug-selective pressure. As the number of patients infected with non-B subtype viruses who are treated with antiretroviral drugs are increasing, understanding the impact of genetic subtype variations on drug resistant evolution has become very important. HIV-1 subtype C is the dominant variant that is driving the epidemic in Southern Africa. The aim of this study was to analyze the impact of genomic diversity at all sites known to be associated with resistance to each of the three classes of antiretroviral drugs protease inhibitors (PIs), nucleoside reverse transcriptase inhibitor (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) on the genetic barrier in subtype primary C viruses isolated from Limpopo Province in South Africa. Since HIV-1 subtype C is responsible for the epidemic in South Africa, there is need to have information on how it compares with subtype B on which the ARVs were developed. To the best of our knowledge, this is the first study of its kind in South Africa and it could generate information that will be of importance in therapy formulation.

MATERIALS AND METHODS

Samples were obtained from HIV-1 infected drug naive patients who were attending voluntary testing and counselling (VCT) and antenatal clinics in Limpopo Province of South Africa. All the patients were adults of 18 years and above and were all infected through heterosexual intercourse. The polymerase gene regions of the viruses were amplified in our ongoing HIV diversity study in Limpopo Province and the genetic barrier that exists in the 35 protease and 44 RT subtype C sequences derived from the isolates were determined. The GenBank accession numbers of the sequences are: GU201798 to GU201826 (protease nucleotide sequences) and GU201754 to GU201797 for the reverse transcriptase genes nucleotide sequences.

Drug resistance mutational analysis

Analysis of drug resistance related mutations in the RT and PR genes was performed using the Stanford HIV Drug Resistance Interpretation Algorithm (www.hivd.stanford.edu/hiv). This interactive program, based on subtype B consensus, compares codons of query sequences with resistance coding nucleotides contained in the database. Also, the sequences were compared for evolution to the drug-resistance-associated substitutions specified by the International AIDS-Society (IAS)-USA 2008 update.

Genetic subtyping of the isolates

The genetic subtypes of the viruses was determined by phylogenetic analysis of the pol sequences and were confirmed to be of subtype C as they intermingled with other subtype C reference sequences obtained from the Los Alamos database (www.hiv.laln.gov).

Determination of genetic barrier for drug resistance substitutions in test isolates

Genetic barrier is an important factor for the development of drug resistance and it is influenced by the number and type of nucleotide mutations (transitions and transversions) required for the evolution from wild-type to drug-resistance associated substitution. The type of nucleotide mutation influences the genetic barrier as transitions (replacement of a purine by another purine: A to G; or pyrimidine by another pyrimidine C to T) are for steric reasons more likely to occur frequently than transversions (the replacement of a purine by a pyrimidine and vice versa; A to C, A to T, G to C, G to T). Nucleotide sequence analysis of HIV-1 PR and RT gene was performed to identify changes related to evolution to drug resistance. Sequences that contained individual resistance codons were excluded from the analysis. The protease sequences contained 99 codons with the exception of one that had an insertion, while the reverse transcriptase (RT) sequences contained 300 codons. The first 300 codons were considered because all the

Table 1. Prevalence of wild-type (WT) codons in test isolates and its impact on genetic barrier at major protease drug-resistance associated positions in antiretroviral naive patients.

Codon	Substitution	Codon in subtype B consensus sequence	Polymorphisms in WT codon subtype C isolate	Prevalence of wild-type codon in test isolate (n = 35)	Closest mutational-resistant codon	Required substitution
30	D30N	GAT	GAT	33	AAT	1 ts
			GAC	2	AAC	1 ts
46	M46I	ATG	ATG	35	ATA	1 ts
	M46L				C/TTG	1tv
48	G48V	GGG	GGA	35	GTA	1 tv
50	I50L	ATT	ATT	35	CTT	1 ts
	I50V		ATT	35	GTT	1 tv
82	V82A	GTC	GTC	34	GCC	1 ts
	I82A		ATC	1	GCC	2 ts
	V82F		GTC	34	TTC	1 tv
	I82F		ATC	1	TTC	1 tv
	V82S		GTC	34	TCC	1 ts
	I82S		ATC	1	TCC	1 ts
	V82T		GTC	34	ACC	2ts
	I82T		ATC	1	ACC	1 ts
84	I84V	ATA	ATA	35	GTA	1 ts
90	L90M	TTG	TTG	35	ATG	1 tv

ts, Transitions; tv, transversion. The numbers designate the number of transitions/transversions required.

known resistance mutations to NRTIs and NNRTIs lie within this region. Sequences were compared for evolution to drug-resistance-associated substitutions specified by the International AIDS-Society (IAS)-USA, update of 2008 and the Stanford HIV Drug Resistance Interpretation Algorithm (www.hivd.stanford.edu/hiv). The resistance associated sites that were considered are D30N, M46I/L, G48V, I50L/V, I/V82A/F/S/T, I82V and L90M for protease inhibitors resistance-associated mutations, while M41L, E44D, A62V, K65R, D67N, T69D, K70R, L74V, V75I, F77L, Y115F, F116Y, V118I, Q151M, M184I, L210W, T215F and K219E/Q for NRTIs were considered as associated sites. For NNRTIs resistance associated sites, L100I, K103N, V106A/M, V108I, Y181C, Y188C/H/L, G190A/S, P225H, M230L and P236L were considered.

RESULTS

This study was based on 35 PR and 44 RT sequences all of which were of HIV-1 subtype C as determined by phylogenetic analysis. There were no sequences with major drug-resistance-associated substitutions. Comparison was based on subtype B consensus sequence in the determination of genetic barrier.

Genetic barrier to protease inhibitor-associated resistance

Most codons were remarkably conserved among the isolates on positions associated with major protease substitutions in subtype B viruses. Only at position 82 was a codon identified that made impact on the genetic barrier. Here, one isolate had an isoleucine (ATC) when compared with valine (GTC) in the rest of the isolates. The shortest distance to a resistance substitution in this isolate was the I82T (ATC to ACC) substitution, whereas the I82A (ATC to GCC) required two transitions, while for V82F, I82F, V82S and I82S (GTC/ATC to TCC), one transversion is needed for evolution of resistant virus. For V82T substitution (GTC to ACC) to occur, two transitions were required for a major resistance substitution at this codon. For other resistance-substitutions in other major resistance codons, one transition was needed to overcome the genetic barrier to protease inhibitors resistance. At position M46, two substitution routes were possible, M46L or M46I. A transversion was needed for M46L (ATG to C/TTG) and a transition was needed for M46I (ATG to ATA) resistance evolution to occur. Also, L90M required one transversion (TTG to ATG) for resistance development to evolve at this codon. Details of the protease resistance associated positions and required type and scope of substitution for resistance to develop are shown in Table 1.

Genetic barrier to NRTI associated resistance

There was no significant difference observed among the isolates at the NRTI resistance-related codons. These differences at the nucleotide level did not affect in general

the genetic barrier for evolution of NRTI resistance-associated substitutions. For T69D, two substitutions: one transition and one transversion each was needed for all the isolates to mutate from the wide type codon ACT/ACC to GAT or GAC. For V118I, which is polymorphic, three isolates needed two substitutions each (GTC/A/G to ATT) to overcome the barrier needed for drug resistance evolution, while the remaining 41 (GTT to ATT) needed just one transition for drug resistance to develop. The highest genetic barrier to NRTI evolution was observed at codon Q151M. At this position, nine isolates required two transversions and one transition each to mutate the wild-type codon CAA to ATG, while 35 (CAG to ATG) required two transversions to overcome the genetic barrier at this codon. Most of the isolates (31) had an increased genetic barrier for evolution to the L210W substitution. The genetic barrier was higher in most subtype C viruses than in the subtype B consensus sequence. These isolates contained either the CTG or the TTA polymorphism at this position. Both codons required one transition and one transversion (CTG or TTA to TGG or TGG) for resistance mutation to occur. Also at T215F, 43 of the isolates had ACC or ACT polymorphism and one transition and one transversion was needed for resistance mutation to develop as in the subtype B consensus reference sequence (Table 2).

Genetic barrier to NNRTI associated resistance

Analysis of the NNRTI resistance mutations showed that L100I which is polymorphic for subtype C at the wild-type codon had differential genetic barriers to overcome before resistance substitution could occur. Depending on the nucleotide that wass present at the codon, if TTA or CTA is present, only one transversion was needed for evolution to drug resistant virus (C/TTA to ATA), whereas if TTG or CTG codon was in place, two substitutions (1tv and 1 ts) were needed for drug resistance mutation development (CTG to ATA). At position 106, there are two codons that could code for valine; CTA or GTG, and there were also two pathways for evolution to resistance viruses: V106A or V106M. One transition was needed for V106A to evolve if codon GTA or GTG was present, while the evolution of V106M via GTA required two transitions (GTA to ATG). Subtype C is predominantly GTG at this position as the majority of the isolates had this codon, thus suggesting that this codon is predominant in C viruses (Brenner et al., 2003).

For V108I, two isolates had GTG which increased genetic barrier to drug resistance mutation with two transitions to substitute GTG to GTA which is one additional substitution when compared to majority of the isolates that only need a single transition to evolve from GTA to ATA.

At position Y188, there are two possible codons coding for the amino acid tyrosine (TAT or TAC). The substitution Y188L required two transversions for resistance development and for Y188C (TAT/C to TGT/C), one transition is needed, while for Y188H (TAT to CAC) two transitions are needed for evolution into resistant viruses. Similarly, G190S (GGA to AGC/TC) required one transition and one transversion to overcome the genetic barrier to drug resistance evolution, while for G190A from GGA to GCA, only one transversion was required, whereas two transversions were needed in three isolates that had the GGG codon (GGG to GCC).

At position P225, one isolate had codon CCA which required two transversions for evolution to the drug resistance associated mutation (CAT or CAC) which codes for histidine, while the majority (43 isolates) had CCT or CCC which needs only one transversion for drug resistance evolution to occur. The remaining NNRTIs resistance codons: K103N and P236L required one transversion each for evolution into drug resistant viruses (Table 3).

DISCUSSION

Genetic barrier is an important factor for the development of HIV drug resistance. Because of genetic barrier variability in HIV-1, particular subtypes could have different genetic barriers for drug resistance substitution (van de Vijver et al., 2006). The genetic barrier observed among the isolates in this study obtained from drug naive patients is the same as those found in other subtype C viruses.

In the protease, I/V82A/T had an increased genetic barrier in the subtype C viruses investigated as compared to other resistant codons with two substitutions for drug resistance to evolve. For other resistance codons, one substitution each is needed to evolve to drug resistance. Bearing in mind that transversion is less likely to occur than transition due to steric reason, L90M, I50V and G48V have higher genetic barrier than D30N, M46I and I50L which require one transition for drug resistance evolution to occur.

Among the NRTI resistance-associated substitutions, an increased genetic barrier was found at positions T69D, V118I, Q151M, L210W and T215F. The V118I substitution occurring in only two isolates will probably have no significant impact on drug susceptibility. The Q151M substitution which is part of a multi-NRTI resistance complex that is associated with resistance to all NRTIs, had the highest genetic barrier as compared to all the NRTIs positions. For drug resistance to evolve in all the earlier mentioned positions, at least two substitutions: two transversions or one transition and two transversions, respectively are required. All the other NRTIs resistance-associated substitutions needed only one substitution each for resistance to evolve.

For NNRTI resistance-associated substitutions, a differential barrier was found for V106M and V106A where

Table 2. Prevalence of wild-type (WT) codons in test isolates and its impact on genetic barrier at NRTI drug-resistance associated positions in antiretroviral naive patients.

Codon	Substitution	Codon in subtype B consensus sequence	Polymorphisms in WT codon (subtype C isolate)	Prevalence of wild-type codon in test isolate (n = 44)	Closest mutational-resistant Codon	Required substitution
41	M41L	ATG	ATG	44	C/TTG	1 tv
44	E44D	GAA	GAA	44	GAC/T	1 tv
62	A62V	GCC	GCC	39	GTC	1 ts
			GCT	4	GTT	1 ts
			GCA	1	GTA	1 ts
65	K65R	AAA	AAA	2	AGA	1 ts
			AAG	42	AGG	1 ts
67	D67N	GAC	GAC	40	AAC	1 ts
			GAT	4	AAT	1 ts
69	T69D	ACT	ACT	41	GAT	1 ts, 1 tv
			ACC	2	GAC	1 ts, 1 tv
70	K70R	AAA	AAA	41	AGA	1 ts
			AAG	3	AGG	1 ts
74	L74V	TTA	TTA	38	GTA	1 tv
			TTG	3	GTA	1 tv
			CTA	3	GTA	1 tv
75	V75I		GTA	44	ATA	1 ts
77	F77L	TTC	TTC	41	CTC	1 ts
			TTT	3	CTT	1 ts
115	Y115F	TAT	TAT	44	TTT	1 tv
116	F116Y	TTT	TTT	43	TAT	1 tv
			TTC	1	TAC	1 tv
118	V118I	GTT	GTT	41	ATT	1 ts
			GTC	1	ATT	2ts
			GTA	1	ATT	1ts, 1tv
			GTG	1	ATT	1ts, 1tv
151	Q151M	CAG	CAG	35	ATG	2 tv
			CAA	9	ATG	1 ts, 2 tv
184	M184I		ATG	44	ATA	1 ts
210	L210W	TTG	TTG	13	TGG	1 tv
			TTA	28	TGG	1 ts, 1tv
			CTG	1	TGG	1 ts, 1tv
			CTA	2	TGG	2 ts, 1tv
215	T215F	ACC	ACC	43	TTC	1 ts, 1 tv
			ACT	1	TTT	1ts, 1 tv
219	K219E/Q	AAA	AAA	3	GAA	1 ts
			AAG	41	GAG	1 ts

*ts, Transition; tv, transversion. The numbers designate the number of transitions/transversions required.

there was a reduced genetic barrier in the test isolates as in other subtype C viruses as compared to other subtypes as previously reported by van de Vijver et al. (2006). This is due to predominance of GTG at codon 106 that results in a lower genetic barrier for V106M to evolve. The V106M substitution confers high-level resistance to all NNRTIs (Brenner et al., 2003). For V108I, only two isolates had GTG which increased the

Table 3. Prevalence of wild-type (WT) codons in test isolates and its impact on genetic barrier at NNRTI resistance-associated positions in antiretroviral naive patients.

Codon	Substitution	Codon in subtype B consensus sequence	Polymorphisms in WT codon (subtype Cisolates)	Prevalence of wild-type in test isolate (n = 44)	Closest mutational-resistance codon	Required substitution
100	L100I	TTA	TTA	39	ATA	1 tv
			TTG	5	ATA	1 ts, 1tv
103	K103N	AAA	AAA	40	AAC/T	1 tv
			AAG	2	AAC/T	1 tv
106	V106A	GTA	GTA	3	GCA	1 ts
			GTG	41	GCG	1 ts
	V106M		GTA	3	ATG	2 ts
			GTG	41	ATG	1 ts
108	V108I	GTA	GTA	42	ATA	1 ts
			GTG	2	ATA	2 ts
181	Y181C	TAT	TAT	39	TGT	1 ts
			TAC	5	TGC	1 ts
188	Y188C	TAT	TAT	39	TGT	1 ts
			TAC	5	TGC	1ts
	Y188H		TAT	39	CAC	2 ts
			TAC	5	CAT	1 ts
	Y188L		TAT	39	TTA	2 tv
			TAC	5	TTA	2 tv
190	G190A	GGA	GGA	41	GCA	1 tv
			GGG	3	GCC	2tv
	G190S		GGA	41	TCC	2tv
			GGG	3	AGC	1 ts
225	P225H	CCT	CCT	1	CAT	1 tv
			CCC	42	CAC	1 tv
			CCA	1	CAC/T	2 tv
230	M230L	ATG	ATG	44	C/TTG	1 tv
236	P236L	CCT	CCT	44	CTT	1 tv

ts, Transition; tv, transversion. The numbers designate the number of transitions/transversions required.

genetic barrier to drug resistance evolution having two transitions to substitute GTG to GTA, which is one additional substitution when compared to majority of the isolates that only needed one single transition to evolve from GTA to ATA. The substitution Y188L required one transition and one transversion for resistance development, while Y188C and Y188H required only one transition in each case to evolve into drug resistance. An increased genetic barrier was observed in A190S where one transition and transversion each was needed for drug resistance evolution. At position P225H, one isolate had CCA which required two transversion for evolution to drug-resistance associated substitution CAT/C which codes for histidine, while 43 isolates had CCT/C which needed only one transversion for resistance development. The other NNRTIs resistance codons: K103N, G190A and P236L required one transversion each for resistance evolution.

Although genetic barrier is an important determinant for emergence of drug resistance, other factors, however, also contribute to the development of resistance. These include viral (genetic background such as mutations outside protease and RT, host cell tropism) and host factors (immune control and target cell availability) (Nijhuis et al., 2001; Theys et al., 2010). The genetic barrier observed was similar for both subtypes C and B at almost all positions that are related to drug-resistance, except at codon V106M where a lower genetic barrier was found in subtype C viruses. Turner et al. (2004) compared nucleotide substitutions and polymorphisms at codons known to confer resistance in subtype B and their findings also identified the GTG polymorphism at RT position 106 of subtype C viruses. Brenner et al. (2003) had previously described a V106M mutation in samples from three patients infected with a subtype C virus which had failed therapy with efavirenz. Also Morris et al. (2003) have shown that this mutation is also seen in subtype C viruses derived from patients who have failed therapy with nevirapine. The selection of this mutation in subtype C viruses results from a single nucleotide change GTG to ATG. Similarly, van de Vijver et al. (2006) also observed that the genetic barrier in a study involving nearly 2000 protease and RT sequences were remarkably similar for all subtypes at all positions that are related to drug-resistance. In addition, they also observed that in the few positions where differences were found, a higher genetic barrier was frequently calculated for some individual non-B subtypes. In this study, we did not observe any major difference in genetic barrier between subtype C and the reference subtype B consensus sequence in all positions that are related to drug resistant mutations.

In conclusion, we found only limited differences in the genetic barrier for evolution to major drug-resistance-associated substitutions between subtypes B and C. These results suggest that subtype variability is not a major influence on the patterns of resistance substitutions that will emerge under drug selective pressure.

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