Antiviral Research 83 (2009) 118–126

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Genotypic analysis of the protease and reverse transcriptase of non-B HIV type 1 clinical isolates from naïve and treated subjects☆

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ARTICLE INFO

Article history:
Received 6 August 2008
Received in revised form 25 March 2009
Accepted 2 April 2009

Keywords:
HIV-1
Non-B subtypes
Resistance

ABSTRACT

One hundred and ninety-two *pol* sequences of drug-naïve and drug-experienced subjects infected with non-B HIV-1 subtypes were analyzed to identify treatment-related amino acid changes which might be relevant for drug-resistance and possibly not included in the accepted mutation list for the B subtype. The correspondence analysis identified non-B-specific and subtype-specific polymorphisms which should not be mistaken for mutations. Multiple χ^2 were performed to detect the differences between naïve vs treated subjects and between different subtypes. To verify the contribution of each single mutation to the resistance levels as predicted by the *Virtual Phenotype*™—LM, simple univariate linear regression was used with fold resistance as a dependent variable and individual mutations as predictors. Commonly accepted protease (PR) and reverse transcriptase (RT) positions along with mutants at RT positions 118 and 90 were significantly associated with treatment. Two unusual PR (K14R and I66F) and five RT positions (E28K, S68G, H221Y, L228R/H and P294A) were also associated with treatment ($p < 0.01$). Only minimal variations were observed with respect to commonly accepted amino acid changes. All amino acid changes correlated with treatment influenced the resistance levels to each single drug. Our findings demonstrate that there are no substantial differences regarding known resistance-associated mutations and the newly emergent substitutions between non-B and B subtype strains.

1. Introduction

Information regarding resistance to antiretroviral drugs is principally derived from patients infected with HIV-1 subtype B (Rhee et al., 2004). However, the spread of non-B viruses is now increasing in previously clade B homogeneous areas such as Italy (Balotta et al., 2001; Monno et al., 2005) where access to HIV therapy is unrestricted; moreover, antiretroviral drugs have been introduced into developing countries representing the cradle of the largest assortment of non-B subtypes. Therefore, it is crucial to acquire further knowledge concerning anti-HIV drug-resistance in non-B strains.

Although patients with non-B HIV infection have been reported to have a significantly lower CD4-cell increase than the B counterpart after initiating HAART (De Wit et al., 2004), generally speaking, there is no evidence of significant differences in virological or immunological response to combination antiretroviral therapy between patients infected with B and non-B subtypes (Bannister et al., 2006), thus indicating that subtype does not affect drug efficacy, at least in first-line therapy (Bocket et al., 2005). However, these observations do not signify that the molecular mechanisms of antiviral resistance in non-B viruses are the same as those in B viruses. Most of the resistance-associated mutations (RAMs) in subtype B viruses are found in treated patients harbouring non-B subtypes (Kantor et al., 2005), thus suggesting similar resistance pathways. Nevertheless, naturally occurring amino acid polymorphisms in non-B subtypes, such as the protease (PR) M36I mutation, are associated with an increased rate of protease inhibitor (PI) failures in the B subtype (Perno et al., 2001). Moreover, non-B subtypes demonstrate more minor PR substitutions than B subtypes (van de Vijver et al., 2005) which, even if they do not impair drug susceptibility by themselves, they could contribute to resistance by increasing the replicative capacity of the resistant virus (Holguín et al., 2006) and also alter the genetic barrier for resistance to new PIs such as tipranavir (TPV) (Vandamme et al., 2005), the muta-

☆ Sponsorship: The study was partly supported by the Ministero della Salute – Rome, Italy.

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doi:10.1016/j.antiviral.2009.04.001

tional score of which includes many mutations naturally occurring in non-B viruses. Moreover, non-B subtypes may also differ in codon usage at sites critical to resistance (Dumans et al., 2004); therefore, certain mutations occur less frequently at least in some non-B subtypes (Gupta et al., 2005; van de Vijver et al., 2006) while subtype C has a particular propensity for the development of K65R (Brenner et al., 2006).

Herein, we analyzed the PR and reverse transcriptase (RT) sequences of naïve and drug-experienced subjects with non-B HIV infection. The objective of the present study was the identification of treatment-related amino acid changes in non-B strains which might be relevant for resistance to current antiretrovirals and which are not included in the accepted mutation list for B subtype. For mutations significantly associated with treatment, we also attempted to evaluate their potential contribution to the resistance level.

2. Materials and methods

2.1. Study population and specimens

A total of 164 drug-naïve and drug-experienced subjects chronically infected with non-B viruses were included in the study. The naïve patients had never been exposed to antiretroviral drugs while those drug-experienced were receiving RT inhibitors (RTIs) and/or PIs at the time of sampling. Plasma HIV-1 RNA was extracted, reverse transcribed, amplified, and sequenced directly (Viroseq™ HIV Genotyping Kit, Celera Diagnostics, Alameda, CA) according to the manufacturer's instructions on an ABI-310 automated sequencer (Applied Biosystems) to obtain a 1260-kb sequence. Dedicated software (HIV Genotyping System Software™, Applied Biosystems, Foster City, CA) was used to analyze the data. Multiple sequences from the same patient were considered if obtained at the time when the patient was still untreated or after each different treatment regimen. Sequences were grouped according to their assigned subtype and treatment status (drug-naïve or drug-experienced); when there was only one or a limited number of non-B sequences available, these were classified as "other".

Ninety-six complete PR and RT sequences from consecutive naïve subjects infected with subtype B HIV-1 virus were used as controls for comparison with those from naïve non-B individuals.

2.2. Subtype assignment

The neighbor-joining method was used to compare the pol sequences (1260 nt) to reference strains of known subtype derived from the Los Alamos database (www.hiv.lanl.gov). Pairwise distance matrices were generated using the Kimura 2-parameter distance estimation method with a transition/transversion ratio of 2.0. The reliability of the phylogenetic clustering was tested using bootstrap analysis with 1000 replicates. Bootstrap values above 70 were considered sufficient for subtype assignment.

2.3. Mutation definitions

Each sequence was translated and compared to the HXB2 reference sequence (accession number, K03455). All positions within the PR and RT were analyzed and differences from the wild-type B sequence were recorded. Mutations were deemed as RAMs if included in the 2008 IAS-USA mutation list (Johnson et al., 2008) and designated as "known" mutations. Major and minor PI-RAMs were defined according to this list (Johnson et al., 2008); "primary" non-nucleoside RT inhibitors (NNRTI)-RAMs refer to mutations associated with first-generation NNRTIs (efavirenz, EFV and nevirapine, NVP) (Johnson et al., 2008). For purpose of analysis, mixtures of mutant and wild-type viruses were considered as mutants. Polymorphisms were defined as mutations which occurred in ≥5% of sequences from naïve subjects (non-B and B-specific polymorphisms) compared to the HXB2 sequence. Subtype-specific polymorphisms were defined as those significantly more prevalent in each non-B subtype from naïve patients.

Resistance was interpreted using the last generation of Virtual Phenotype (*Virtual Phenotype*™—LM; Virco lab) which accurately predicts real phenotype resistance (Perez-Elias et al., 2003); resistance levels to the single drugs were expressed as the predicted fold change (FC).

2.4. Statistical analysis

Multiple χ^2 tests were performed to detect differences in the frequency of amino acids at specific positions between different subtypes, and between naïve vs treated persons. As this is an exploratory analysis, rather than performing formal p-value correction, any differences with $p < 0.01$ were considered potentially relevant.

Correspondence analysis was used to identify a possible relationship between polymorphisms in naïve subjects and individual HIV subtypes. Correspondence analysis is a descriptive, exploratory, multivariate analysis, whose main aim is to represent a set of data by points in a multidimensional space, providing a visual interpretation of patterns in the data (Greenacre, 1992). This method allows one to identify a space reduced to only a few dimensions, in which the structure of the data is represented in a fairly significant manner that is not discordant with the real multidimensional structure of the data distribution. In other terms, the relative position between points represents the probability of an independent association between the considered variables. Frequency matrices are calculated and, in order to understand the data overall and the interrelationships between the different groups, correspondence analysis was carried out for each matrix. For this analysis, basic data were expressed as presence/absence of individual polymorphisms, and HIV subtype. The data were arranged in matrices in which the rows represented individual mutations. Only polymorphisms with a different distribution among subtypes in a previous exploratory analysis were included. As correspondence analysis is an exploratory technique, no p-values could be obtained.

To verify the contribution of each single mutation to the drug-resistance levels as predicted by the *Virtual Phenotype*™—LM, simple univariate linear regression was used. For this purpose, fold resistance to the single drug was log transformed and used as a dependent variable while individual mutations were the predictors. For the sake of clarity, results with log folds were exponentiated again to show clinical usefulness measure. All positions which significantly ($p < 0.01$) contributed to modifying resistance levels to single drugs were recorded. However, in subsequent analyses, for each drug, only known positions and unreported positions significantly associated to treatment were retained.

2.5. Ethics

The study did not require approval from the ethics committee since it was a retrospective study in which the clinical data and the HIV pol sequences were obtained from plasma samples used for clinical routine.

3. Results

3.1. Population and sequence data

The 164 subjects of non-Italian (*n*. 118) and Italian origin (*n*. 46) included 49 females and 115 males with a median age of 33.8

Table 1
Principal data associated with the 190 protease and 192 reverse transcriptase sequences obtained from patients infected with non-B HIV-1 variants either naïve and treated with antiretroviral therapy.

	Naïve	Treated	*p*-value[a]
PR sequences	94	96	
RT sequences	95	97	
Subtype			
A/AE	12	17	0.34
02_AG	27	39	0.08
C	27	8	0.0003
F	11	16	0.32
G	10	7	0.42
Other	4	7	0.37
Unassigned	4	3	0.48
Median CD4+ (IQR[b])	199 (100.5–304.5)	235 (124–321)	0.11
Median pVL (IQR[b])	52,800 (9,000–146,050)	7,100 (1,500–54,255)	0.0002
ART regimen[c]			
2 or 3 NRTIs[d]		7 (8.1%)	
2 NRTIs + PI[e]		31 (36%)	
2 NRTIs + NNRTI[f]		42 (48.8%)	
3 class		6 (7.0%)	
Median duration of ART (months) (range)	NA[g]	12.7 (2.8–74.8)	
Mean ± SD[h] number of total PR mutations (range)	9.41 ± 2.1	11.54 ± 4.1	0.0003
Mean ± SD[h] number of total RT mutations	19.81 ± 5.09	23.86 ± 5.3	<0.0001
Patients with major PI mutations, *n.* (%)	0	29 (30.8%)	<0.0001
Patients with primary RTI mutations, *n.* (%)	1 (1%)	72 (75%)	<0.0001
Patients with primary NNRTI mutations, *n.* (%)	2 (2.1%)	50 (51.5%)	<0.0001

[a] The χ^2 test or Fisher exact test and the Mann–Whitney test were used as appropriate.
[b] Interquartile range.
[c] Antiretroviral treatment was known for 86 patients.
[d] Nucleos(t)ide reverse transcriptase inhibitors.
[e] Protease inhibitors.
[f] Non-nucleoside reverse transcriptase inhibitors.
[g] Not applicable.
[h] Standard deviation.

years (min–max, 22–60), the majority of whom (89%) acquired their HIV infection through sexual contact. These 164 patients furnished 192 valuable sequences including 28 sequences from 18 patients who had serial sequences obtained either before and after treatment initiation, or after different treatment regimens. The 192 pol sequences comprised 190 complete PR (aa 1–99) and RT (aa 1–300) sequences plus two sequences which included RT alone. Ninety-four PR and 95 RT sequences corresponded to naïve patients, while 96 PR and 97 RT sequences were obtained during the course of antiretroviral therapy. Data associated with the PR and RT sequences from naïve and treated patients are listed in Table 1.

3.2. HIV-1 subtypes

The distribution of subtypes among the 164 patient was as follows: 10 A1 (6.09%), 17 CRF01_AE (10.3%), 51 CRF02_AG (31.0%), 33 C (20.1%), 19 F1 (11.5%), 17 G (10.3%), and 10 (6.09%) which were classified as "other" (one each for subtype J, BF and 10_CD, two each for D and 14_BG subtypes, and three for 06_cpx subtype). The subtype was unclassifiable in seven (4.2%) patients. As CRF01_AE isolates do not contain recombinant breakpoints within the *pol* gene and the entire *pol* region corresponds to subtype A (Carr et al., 1996; Gao et al., 1996) (discordance between consensus A and AE partial pol sequences = 4.26%), sequences classified as A and CRF01_AE subtypes were grouped together (A/AE). Therefore, the 190 complete sequences included: 29 A/AE sequences, 66 CRF02_AG, 34 C, 27 F, 16 G, 11 "other", and seven unclassified (Table 1). The two sequences including the RT alone were C (treated) and G (naïve), respectively.

3.3. Treatment regimen

The treatment regimen at the time of genotyping was known for 86/97 (88.6%) sequences obtained during treatment (Table 1). A total of 8.1% sequences were obtained from patients receiving 2–3 nucleos(t)ide RT inhibitors (NRTIs), as compared to 36% and 48.8% during a PI- or a NNRTI-based regimen, respectively, and 7.0% during a triple-class regimen. Overall, the median duration of antiretroviral therapy was 12.7 months (min–max, 2.8–74.8). Nineteen (22%) patients had received >1 treatment regimen, while 25.5% of sequences were obtained at the time of first therapy failure.

3.4. Polymorphisms in naïve patients

Natural sequence polymorphisms were detected at 27 PR and 46 RT positions of non-B strains. However, after analyzing the sequences from B subtype naïve patients, the polymorphisms significantly associated with non-B subtypes ($p < 0.01$) were 13V, 15V, 20I/R, 35D/Q, 36I, 41K, 43R, 69K, 70R, 82I, and 89 M within the PR and 11T, 35T/K, 36D/A, 39E/K/A/D, 40D, 43E/R, 49R, 122E, 158S, 162A/C, 173T/A, 174K, 177E, 200A, 207E/A/D/N, 248D/N, 250E, 286A, 291D, 293V, 294T within the RT. Conversely, substitutions at positions 37, 63 and 64 in the PR, and 142, 196, and 297 in the RT were associated with B subtype ($p < 0.01$). At correspondence analysis, a possible association with individual subtypes were observed for PR polymorphisms I13V, I15V, K20I/R, E35D/Q, H69K, V82I and L89 M (Fig. 1, top). For example, I13V was more likely to occur in CRF02_AG, A/AE and G subtypes; I15V in C and F1 subtypes; K20I in CRF02_AG and G subtypes, and K20R in F1, whereas wild-type (wt) K20 was observed in A/AE strains. As expected, V82I was associated with G subtype (Vergne et al., 2000). Of note, subtype F carried a wt

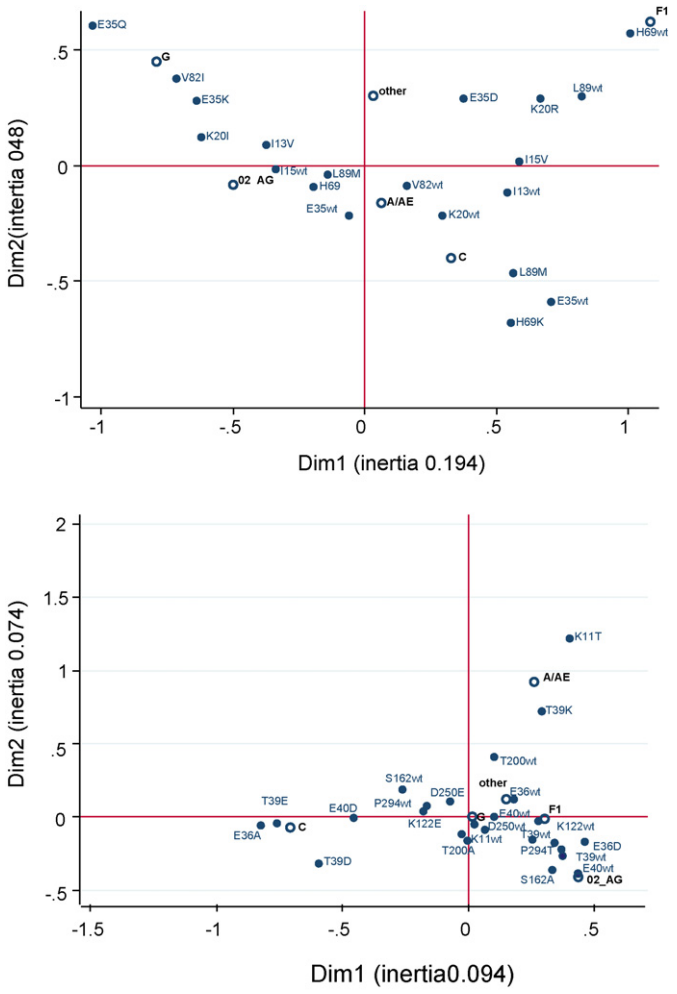

Fig. 1. Correspondence analysis between HIV non-B subtypes and individual polymorphisms in the protease (top) and reverse transcriptase (bottom). Correspondence analysis is a descriptive, exploratory, multivariate analysis whose aim is to represent a set of data by points in a multidimensional space, providing a graphic interpretation of data patterns. Basic data are expressed as presence/absence of individual polymorphisms, and HIV subtype; the relative position of points represents the probability of an association between the variables: the closer the points, the more likely is an association. The overall variability in the data is defined as "inertia". In this figure, only polymorphisms (full circles) with a possible association with individual subtypes (empty circles) are shown. wt = wild-type.

H69 and was more likely to carry a wt L89 than other subtypes. Within the RT, differences were found in the distribution of the K11T, E36D/A, T39E/K/A/D, E40D, K122E, S162A, T200A, D250E, and P294T polymorphisms (Fig. 1, bottom).

3.5. Treatment-related mutations in non-B naïve group

Naïve non-B subjects had a statistically significant lower number of total PR and RT mutations than treated patients ($p = 0.0003$ and $p < 0.0001$, respectively) (Table 1). No major PI-RAM was detected; one patient (1%) had the K219Q thymidine-analogue mutation (TAM), while primary NNRTI-associated mutations (K103N and V108I) were detected in two patients (2.1%, one patient each). Other mutations in naïve subjects were: T69A and L210V or E (2%, each), V106I (4.2%), Y181P (1.0%) and V179D (1%) or V179I (8.4%).

3.6. Protease mutations in non-B treated subjects

After excluding non-B-specific polymorphisms, amino acid changes at resistance-associated positions were observed in 83 (86.4%) sequences with a median of 0 (min–max, 0–6) and 2 (min–max, 0–8) mutations at major and minor sites, respectively. Patients on PI-based regimens and those receiving a triple-class regimen had a significantly higher number of mutations ($p = 0.0001$, $p = 0.0017$, respectively) than patients on NNRTI-based regimens and those treated with 2 or 3 NRTIs.

Fig. 2 (top) demonstrates PR differences between HIV-1 isolates from treated and naïve persons infected with a non-B subtype. In this figure, all positions in the IAS list and those with significant differences are reported for any amino acid change. Known PR positions significantly associated with treatment exposure were 10, 13, 33, 46, 54, 71, 74, 84, and 90. Two unreported positions, 14 and 66, were also associated with treatment ($p < 0.01$).

3.7. Reverse transcriptase mutations in non-B treated subjects

Amino acid changes at any NRTI-associated position were detected in 78.3% of treated patients, and M184 mutants were noted in 58.7%. In 70.1% of cases, mutations at positions associated with NNRTI resistance were observed. The median number of mutations at positions associated with resistance to NRTIs and NNRTIs was 2 (min–max, 0–8) and 1 (min–max, 0–5), respectively. Sequences from patients on a triple-class regimen had a statistically significant higher number of NRTI ($p = 0.0082$) and NNRTI mutations ($p = 0.0011$).

Mutations significantly associated with treatment were at known NRTI positions 41, 67, 70, 74, 75, 184, 215, and 219, and NNRTI positions 100, 103, 108, 181, 190 and 225. In addition, mutants at positions 118 (currently excluded from the IAS list), and 90 included in the etravirine score (Johnson et al., 2008), were also statistically associated with treatment. Unusual treatment-related positions were 28, 68, 221, 228 and 294 ($p < 0.01$) (Fig. 2, bottom).

3.8. Amino acid changes in sequences from non-B treated patients

Table 2 lists all the amino acid changes in sequences from treated patients. Only positions included in Section 2 and those significantly associated with treatment are considered. Twenty-nine patients (30.8%) had at least one major PI-RAM and 15/29 (51.7%) had >2 major PI-RAMs. The prevalence of major PI-RAMs was as follows: L33F 9.3%, M46I/L 12.5%, I47A/V 3.1%, G48V and I54M 1.0% each, L76V 4.1%, V82A 13.5%, I84V 8.3%, N88S 3.1%, L90M 9.3%. Only minimal variations (i.e. G48M in one patient) were observed with respect to commonly accepted amino acid changes. Mutations D30N, V32I and I50LV were never detected. Regarding minor known positions, the largest amino acid variability was observed at codon 63; the I13V polymorphism was replaced with the unreported I13A substitution in eight AG recombinants (20.5%), and the T74A/S mutation prevailed over the known T74P. At the unreported PR position 14, the Lys → Arg change occurred in most sequences while the Ile → Phe substitution occurred at position 66 in 6/6 sequences. A statistically significant different distribution of mutant positions 48, 54, 63, 74, 82 and 93 was noted for the various subtypes.

Typical TAMs were detected in 45 (46.3%) RT sequences from treated patients, 35 of whom displayed >2 TAMs. The M184V mutation was seen in 52 (53.6%) sequences while an additional five sequences (5.1%) had M184I. Overall, atypical TAMs were demonstrated in 13.4% of sequences. The greatest amino acid variability was observed at position 215 in that 27.2% of sequences bearing the T215 mutation had substitutions other than Y or F.

Primary typical NNRTI mutations were detected in 50 (51.5%) sequences with 21 showing >1 NNRTI mutations. Some variability with respect to common amino acid changes was observed at codon 190; in fact, 3/8 sequences had G190E/V/R. The V106A/M mutation was never found.

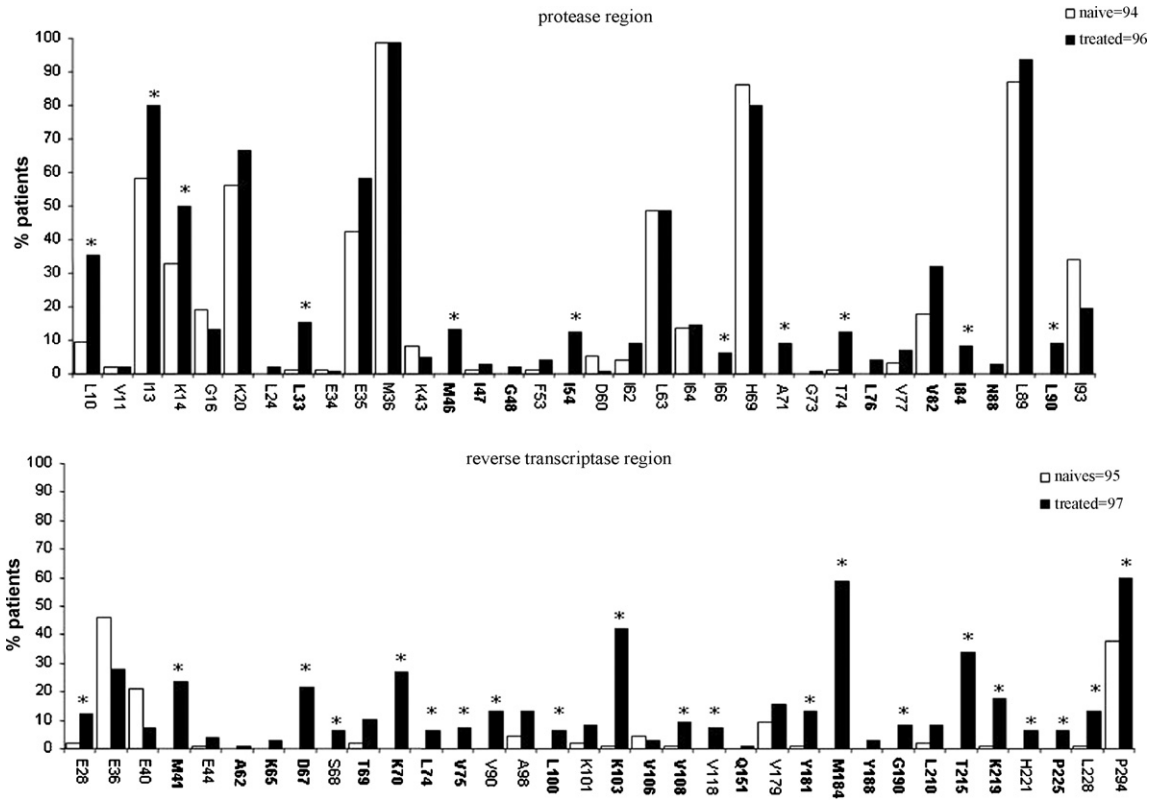

Fig. 2. Proportion of treated patients bearing single protease and reverse transcriptase mutations compared to naïve patients. In this figure, all positions in the IAS list (irrespective of the amino acid change) and unreported treatment-related positions are included. Major protease and primary reverse transcriptase positions are in bold type. *Positions significantly associated with treatment exposure. Polymorphisms at positions 36 and 40 in the reverse transcriptase were significantly less frequent in treated than in untreated persons.

Mutations at unusual positions significantly associated with treatment and the respective prevalence of the specific amino acid change were as follows: E28K (91.7%), S68G (100%), H221Y (100%), L228R (53.8%), L228H (46.2%) and P294A (42.8%) (Table 2). A statistically significant different distribution of mutations at positions 69, 118 and 179 was noted for the various subtypes.

3.9. Levels of resistance to the antiretrovirals according to single unreported mutations

With simple linear regression (Table 3), the presence of the unreported RT E28K mutation appeared to slightly increase resistance levels to zidovudine (ZDV), while S68G increased resistance to didanosine (ddI) and stavudine (d4T), and to a greater extent to lamivudine (3TC) and emtricitabine (FTC). The L228H/R mutation seemed to intensify resistance to ZDV, d4T and tenofovir disoproxil fumarate (TDF) but especially to nevirapine (NVP) and efavirenz (EFV). H221Y augmented resistance to ddI and d4T, but particularly to NVP and EFV, whereas P294A increased resistance to both 3TC and FTC, and, to a lesser extent, to ddI and abacavir (ABC). Lastly, V90I, associated with resistance to next-generation NNRTIs, increased resistance to NVP and especially to EFV.

Within the PR, I66F appeared to particularly increase resistance to boosted darunavir (DRV/r), fosamprenavir (FSV/r), indinavir (IDV/r) and lopinavir (LPV/r), while the presence of K14 mutants determined a slight increase in resistance to DRV/r. The atypical T74A/S appeared to augment resistance particularly to unboosted atazanavir (ATV), and to FSV/r, IDV/r, nelfinavir and boosted saquinavir.

4. Discussion

The susceptibility of different HIV-1 subtypes to antiretroviral drugs is currently the subject of much attention. However, PR and RT sequencing data from treated non-B subtypes are scarce throughout the world (Rhee et al., 2006) and, hence, further research on this topic should be encouraged.

The object of the present study was the identification of treatment-related amino acid changes in non-B strains which might be involved in resistance to current antiretroviral drugs and eventually not included in the accepted mutation list for B subtype.

Overall, our results, although derived from a limited population sample, coincide with those of a previous study (Kantor et al., 2005) in which non-B strains from antiretroviral-treated patients shared drug-resistance positions with the B subtype, and mutations at these positions were significantly associated with treatment exposure. Positions currently excluded from the accepted mutation list (Johnson et al., 2008), such as V118, and newly introduced positions, such as V90 in the RT, were also significantly related to treatment in our series and detected only in treated patients. The V118I substitution occurs as a polymorphism in 2–3% of subtype B naïve patients (http://hivdb. stanford.edu/) (Stanford University, 2007) and, when it is the sole NRTI mutation detected, its presence is not considered relevant; however, V118I has frequently been observed in patients failing antiretroviral therapy (Romano et al., 2002), and a moderate effect on resistance cannot be excluded (Mihailidis et al., 2008). The V90I mutation has been associated with a reduced virological response to TMC125 (etravirine) (Vingerhoets et al., 2007). In our setting, V90I appeared to greatly increase the resistance levels to EFV and, to a lesser extent, to NVP, thereby further complicating clinical management and extending cross-resistance.

Table 2
Amino acid changes detected in sequences from treated patients. Data are shown by subtype, and for both protease and reverse transcriptase only positions included in Section 2 and those significantly associated with treatment are enclosed. Non-B-specific polymorphisms have been excluded. For known positions, atypical amino acid changes are in italic. The number of sequences carrying each mutant amino acid is reported on the right. Positions significantly associated with treatment are bold-typed.

PROTEASE REGION

A/AE (*n.* 17)	02_AG (*n.* 39)	C (*n.* 7)	F (*n.* 16)	G (*n.* 7)	Other (*n.* 7)	Unclassified (*n.* 3)	*p*-value[a]
10I[3]; 10V[2]; *10M*[1]	**10I[5]; 10V[5]**		**10I[7]; 10V[4]**	**10I[2]; 10V[1]**	**10I[1]; 10F[1]**	**10I[1]; *10M*[1]**	0.02
	11F[1]		11I[1]				0.89
	***13A*[8]**						0.04
14R[5]	**14R[21]; 14G[2]; 14SI[1]**	**14R[1]**	**14R[7]**	**14R[5]**	**14R[4]**	**14R[2]**	0.10
16E[1]	16E[3]; *16D*[2]		16E[4]		16E[2]	16E[1]	0.30
20T[1]	20V[3]						0.79
			24I[1]		24I[1]		0.22
	33F[4]; 33I[3]; 33V[3]		**33F[4]**		**33F[1]**		0.11
	34G[1]						
	35G[4]; *35KR*[1]						0.4
46I[1]; 46L[1]	**46I[5]; 46IV[1]**		**46L[5]**				0.22
	47V[2]		47ATV[1]				0.88
48M[1]						48V[1]	<0.01
	53L[3]		53L[1]				0.78
54S[1]	**54V[2]**		**54V[5]**		**54M[1]; 54V[2]**	**54V[1]**	<0.01
	Q58E[4]						0.41
			D60E[1]				0.53
62V[1]	62V[2]		62V[4]		62V[2]		0.10
63P[5]; *63V*[2]	*63A*[1]; *63H*[2]; 63P[8]; *63S*[1]; *63T*[1]	63P[3]; *63S*[1]; *63T*[1]	*63T*[12]; *63S*[1]	*63A*[1]; 63P[3]; *63S*[1]	63P[2]	*63V*[1]; 63P[1]	<0.01
64V[1]	64L[6]; 64M[2]; 64V[1]	64L[1]; 64M[1]	64M[1]; 64V[1]				0.28
	66F[6]						0.15
69KT[1]; *69Y*[1]; *69Q*[1]	*69Q*[1]; *69R*[1]						0.27
	***71G*[1]; 71T[4]; 71V[1]**	**71V[1]**			**71V[2]**		0.15
73D[1]							0.58
***74S*[1]**	***74S*[1]; *74PSA*[1]**		***74A*[5]; *74S*[1]**		**74P[1]; *74S*[2]**		<0.01
	76V[4]						0.41
77I[1]	77I[2]		77I[1]	77I[1]	77I[2]		0.38
82A[1]	82A[1]		82A[9]		82A[2]		<0.0001
	84V[4]		**84V[4]**				0.13
88S[2]	88S[1]						0.47
89V[1]	89V[6]; 89I[2]	89T[1]					0.19
90M[1]	**90M[1]**	**90M[1]**	**90M[5]**		**90M[1]**		0.04
93L[2]	93L[1]	93L[6]	93L[7]		93L[2]	93L[1]	<0.0001

REVERSE TRANSCRIPTASE REGION

A, 01_AE (*n.* 17)	02_AG (*n.* 39)	C (*n.* 8)	F (*n.* 16)	G (*n.* 7)	Other (*n.* 7)	Unclassifiable (*n.* 3)	*p*-value[a]
	28D[1]; 28K[3]	**28K[1]**	**28K[4]**	**28K[2]**		**28K[1]**	0.18
41L[5]	**41L[6]; *41R*[1]**	**41L[1]**	**41L[7]**		**41L[2]**	**41L[1]**	0.27
62V[1]							0.57
65R[1]	65R[2]						0.89
67N[6]; *67G*[1]	**67N[8]**	**67N[1]**	**67N[3]**		**67N[1]**	**67N[1]**	0.34
68G[2]	**68G[4]**						0.34
69D[1]; 69N[2]; 69S[1]; 69DNV[1]	69N[2]				69D[1]	69NI[1]; 69D[1]	<0.01
70R[7]	**70R[11]; 70E[2]; *70G*[1]; *70N*[1]**		**70R[2]**		**70R[1]**	**70R[1]**	0.05
74V[1]	***74I*[2]**		**74V[2]**	**74V[1]**			0.61
75I[1]; *75L*[1]; *75M*[1]	**75L[3]**			***75A*[1]**			0.42
	90I[7]	**90I[1]**	**90I[5]**				0.11
98G[1]	98G[8]	98S[1]		98G[1]	98S[2]		0.33
	100I[1]		**100I[4]**		**100I[1]**		0.03
101P[1]; 101R[1]	101E[2]; 101QR[1]	101Q[1]			101E[1]	101P[1]	0.49
103N[6]	**103N[16]**	**103N[1]**	**103N[5]; *103R*[4]**	**103N[3]**	**103N[4]**	**103N[2]**	0.43
	108I[8]				**108I[1]**		0.08
118I[1]; 118L[1]			**118I[4]**			**118I[1]**	<0.01
151M[1]							0.57
179F[1]; *179I*[7]	*179I*[2]	*179I*[1]	179D[4]				<0.01
Y81C[5]	**181C[2]**		**181C[3]**	**181C[2]**	**181C[1]**		0.14
184V[9]	**184V[29]**	**184V[1]**	**184V[7]; 184I[2]**	**184V[2]; 184I[1]**	**184V[2]; 184I[1]**	**184V[3]**	0.02
	188L[1]			188C[1]	188C[1]		0.29
190A[1]; *190E*[1]	***190V*[1]; 190S[1]**		**190A[2]; *190R*[1]**		**190A[1]**		0.53
210W[1]	210W[2]		210W[4]			210W[1]	0.08
***215D*[1]; 215F[4]; *215N*[1]; 215Y[1]**	***215AP*[1]; *215DE*[1]; 215F[3]; *215S*[1]; *215V*[2]; 215Y[5]**	**215Y[1]**	***215IFS*[1]; 215F[1]; *215S*[1]; 215Y[6]**		**215Y[1]**	**215Y[2]**	0.06
219E[3]; 219Q[3]	**219E[1]; *219G*[1]; *219Q*[6]**	***219R*[1]**	**219E[2]**				0.23
221Y[1]	**221Y[1]**		**221Y[2]**	**221Y[2]**			0.16
	225H[6]						0.14
228H[1]	**228H[4]; 228R[1]**		**228R[4]**		**228H[1]; 228R[1]**	**228R[1]**	0.28
294A[1]; 294H[1]; 294Q[1]	**294A[1]; 294V[1]**		**294S[1]**			**294A[1]**	0.25

[a] χ^2 test

Table 3
Simple univariate linear regression demonstrating the contribution of single mutations to the drug-resistance levels as predicted by the *Virtual Phenotype*™—LM. Only data concerning previously unreported mutations are shown. In this analysis fold resistance to the single drug was log transformed and used as a dependent variable while individual mutations were the predictors. For clarity sake, results with log folds were exponentiated again to show a clinically useful measure.

RT mutation	Drug	Fold increase	95% C.I.		*p*-value
E28K	ZDV	2.23	0.95	5.24	<0.001
S68G	ddI	2.30	1.03	5.15	<0.001
	d4T	1.29	0.6	2.75	0.005
	3TC	14.41	3.65	56.89	<0.001
	FTC	14.81	3.7	59.31	<0.001
V90I	NVP	6.65	2.12	20.89	<0.001
	EFV	15.76	4.45	55.8	<0.001
H221Y	ddI	2.47	1.11	5.51	<0.001
	d4T	1.38	0.65	2.95	<0.001
	NVP	14.81	3.93	55.83	<0.001
	NVP	14.81	3.93	55.83	<0.001
	EFV	12.23	2.67	56.04	0.004
L228H/R	ZDV	2.85	1.22	6.64	<0.001
	d4T	1.31	0.63	2.71	<0.001
	TDF	1.83	0.86	3.9	<0.001
	NVP	10.57	3.53	31.64	<0.001
	EFV	34.68	10.52	114.3	<0.001
P294A	ddI	1.15	0.56	2.37	0.008
	ABC	1.34	0.63	2.86	0.001
	3TC	2.81	1.12	7.05	<0.001
	FTC	2.72	1.08	6.88	<0.001
PR mutation	Drug	Fold increase	95% C.I.		p-value
K14G/R	DRV/r	1.32	0.61	2.85	0.006
I66F	ATV	4.44	1.63	12.04	<0.001
	DRV/r	10.40	4.27	25.34	<0.001
	FSV/r	12.69	4.66	34.55	<0.001
	IDV/r	25.33	9.24	69.41	<0.001
	LPV/r	18.60	6.57	52.62	<0.001
	NFV	5.40	1.88	15.53	<0.001
	SQV/r	4.83	1.76	13.24	<0.001
	TPV/r	2.43	1.02	5.76	<0.001
T74A/S	ATV	6.14	2.55	14.81	<0.001
	DRV/r	2.24	0.95	5.32	<0.001
	FSV/r	4.30	1.71	10.82	<0.001
	IDV/r	4.78	1.86	12.32	<0.001
	NFV	9.41	3.78	23.41	<0.001
	SQV/r	6.29	2.59	15.32	<0.001
	TPV/r	1.84	1,40	2,42	<0.001

ZDV = zidovudine; ddI = didanosine; d4T = stavudine; 3TC = lamivudine; FTC = emtricitabine; NVP = nevirapine; EFV = efavirenz; TDF = tenofovir disoproxil fumarato; ABC = abacavir; DRV/r = boosted darunavir; ATV = atazanavir; FSV/r = boosted fosamprenavir; IDV/r = boosted indinavir; LPV/r = lopinavir; NFV = nelfinavir; SQV/r = boosted saquinavir; TPV/r = boosted tipranavir.

Moreover, we found that amino acid changes at some currently non-recognized positions were also significantly associated with treatment. The K14R PR mutation has been previously reported in naïve subjects from Cameroon and was mainly found among AG viruses but absent in C and F2 subtypes (Konings et al., 2004). Likewise, in our series, K14R was never detected in naïve C and F subtypes (data not shown), whereas 44% of subtype F-treated patients carried this mutation. As the K14 position (along with others) is located in the PR variable region, the possibility that this mutant somehow contributes to PI resistance must be taken into account.

The association between the I66F PR mutation and antiretroviral drug pressure has been ascertained in subtype B isolates (Parkin et al., 2002; Rhee et al., 2005) and associated to cross-resistance between LPV and amprenavir (Parkin et al., 2002); in our patient sample, this mutation appeared to (variably) augment resistance to all PIs, particularly to DRV, FSV, IDV and LPV. The RT E28K, H221Y and L228R/H mutations have been found to be significantly increased in subtype B-treated patients (Ceccherini-Silberstein et al., 2005), with both H221Y and L228R/H which have been linked to NNRTI resistance (Saracino et al., 2006). In our study, mutants L228R/H and H221Y also provoked a significant increase in EFV and NVP resistance. The slight increase of resistance to NRTIs noted with these same mutations might depend on the association of NRTIs with NNRTIs in the current regimens. Lastly, the S68G mutation, which is not just a polymorphism (Røge et al., 2003) and to which a compensatory effect for the K65R mutation in RT has been attributed (Svarovskaia et al., 2008), caused only a minimal change in resistance to d4T and ddI and was associated into K65R in 2/3 of our patients carrying this mutation.

Therefore, it would appear that both the commonly accepted PR and RT resistance-associated positions and the newly emergent positions are shared between B and non-B subtypes.

Regarding the type of mutations at known resistance sites, we observed some amino acid variations with respect to reported changes. The I13V mutation included in the TPV score (Johnson et al., 2008), which occurred naturally among AG and G strains from naïve patients in our series, was replaced with the I13A substitution in eight AG strains from drug-treated patients and appeared to augment the resistance levels to FSV, DRV, LPV and IDV (data not shown). In agreement with a previous observation (McGrath et al., 2007), the PR T74P mutation for TPV resistance (Johnson et al., 2008) was uncommon in our series (16.6%) compared to T74A/S (83.3%). Of note, the T74 codon (ACA) was usually conserved among the different subtypes (data not shown) and the shortest distance to a substitution would be T74A (1 transition from *A*CA to *G*CA). Within the RT, the greatest variability was observed at position 215, with 27% of mutant sequences carrying some "revertant" mutations (Johnson et al., 2008).

The distribution of amino acid changes at known resistance positions showed only a limited variation between subtypes, thereby confirming that the genetic barrier was generally similar for all subtypes at almost all positions (van de Vijver et al., 2006). Statistically significant differences were observed at PR positions 48, 54, 63, 74, 82, 93, and at RT positions 69, 118, and 179. The predominance of the V82A mutation among F strains may be explained by the conserved valine (GTC), thus facilitating the evolution to the 82A (GCC) substitution (1 transition) (van de Vijver et al., 2006); the lack of this mutation among G subtypes is expected since most wild-type G sequences have isoleucine (ATC) (Vergne et al., 2000) which increases the genetic barrier for evolution to the 82A substitution. Even if not statistically significant, subtype F in our series demonstrated a higher propensity to develop L90M than other subtypes. This finding, contrary to previous observations indicating an infrequent development of L90M in subtype F isolates (Parkin et al., 2002), might be explained by the unusual presence of the L89M mutation in our wild-type F strains which increases the genetic barrier thus avoiding the accumulation of the L90M resistance mutation (Calazans et al., 2005).

A higher genetic barrier to the acquisition of L210W has been reported for the F, G and AG subtypes (van de Vijver et al., 2006). In agreement with this observation, 210 mutants were rare among our subtypes without a significantly different distribution ($p = 0.14$). However, L210W was detected in 25% of our F strains; this contradiction might be explained by an extended drug pressure, in that 3/4 F strains bearing L210W were obtained from multi-experienced patients treated with a triple-class regimen.

In this study, the contribution of each single known and newly emergent mutation to drug-resistance levels was evaluated, demonstrating that all positions/mutations significantly associated with treatment variably influenced the resistance levels to each single drug. For this purpose, simple linear regression was

applied using FC predicted by the last generation *Virtual Phenotype* as dependent variable and individual mutations as predictors. Certainly, the availability of real phenotype would have been more appropriate. However, we believe that the *Virtual Phenotype* approach, although arbitrary, is acceptable as it accurately predicts real phenotype resistance (Perez-Elias et al., 2003) by using the genotype–phenotype correlative database representing the currently circulating viral strains to produce phenotypic predictions. Moreover, the most recent *Virtual Phenotype* version weighs the contribution of individual/pairs of mutations to construct FC predictions. The fact that both known and unknown mutations identified in the present and previous studies (Kantor et al., 2005) are shared between B and non-B strains, renders a bias less likely when using the *Virtual Phenotype* methodology, and even though the majority of genotypes/phenotypes in the Virco database refer to B strains, it can be considered equally valid for the analysis of non-B strains. On the other hand, one can object that phenotypic assays for non-B strains in its database have been performed using subtype B vectors, but previous studies have reported that current phenotypic assays can also furnish reproducible results for testing antiretroviral susceptibility in non-B subtypes (Abecasis et al., 2006).

In conclusion, although this exploratory analysis is limited by the small sample size for each subtype and requires confirmation by further studies, it would appear that there were no substantial differences regarding both the known resistance-associated mutations and the newly emergent substitutions between non-B and B subtype strains in our series. This observation signifies that the clinical management and the initial treatment strategies for patients harbouring a non-B subtype, at least, can benefit from adoption of the same treatments and drug-resistance interpretations in B patients. Nevertheless, the continual evolution of non-B subtypes and circulating recombinant forms with the emergence of more complex viruses requires a constant surveillance to avoid that eventually new resistance patterns elude recognition.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

The authors wish to thank Virco BVBA (Mechelen, Belgium) for offering the Virtual Phenotype and Ivo Van Walle for his reading of the manuscript. The authors also thank Mrs Paulene Butts for her assistance in the manuscript preparation and Mrs Eliana Cinori for technical assistance.

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