Original article

HIV type-1 genotypic resistance profiles in vertically infected patients from Argentina reveal an association between K103N+L100I and L74V mutations

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Background: Patterns and pathways of HIV type-1 (HIV-1) antiretroviral (ARV) drug resistance-associated mutations in clinical isolates are conditioned by ARV history and factors such as viral subtype and fitness. Our aim was to analyse the frequency and association of ARV drug resistance mutations in a group of long-term vertically infected patients from Argentina.

Methods: Plasma samples from 71 patients (38 children and 33 adolescents) were collected for genotypic HIV-1 ARV resistance testing during the period between February 2006 and October 2008. Statistically significant pairwise associations between ARV resistance mutations in *pol*, as well as associations between mutations and drug exposure, were identified using Fisher's exact tests with Bonferroni and false discovery rate corrections. Phylogenetic analyses were performed for subtype assignment.

Results: In protease (PR), resistance-associated mutations M46I/L, I54M/L/V/A/S and V82A/F/T/S/M/I were associated with each other and with minor mutations at codons 10, 24 and 71. Mutations V82A/F/T/S/M/I were primarily selected by the administration of ritonavir (RTV) in an historical ARV regimen. In reverse transcriptase, thymidine analogue mutation (TAM)1 profile was more common than TAM2. The non-nucleoside K103N+L100I mutations were observed at high frequency (15.5%) and were significantly associated with the nucleoside mutation L74V in BF recombinants.

Conclusions: Associations of mutations at PR sites reflect the frequent use of RTV at an early time in this group of patients and convergent resistance mechanisms driven by the high exposure to protease inhibitors, as well as local HIV-1 diversity. The results provide clinical evidence of a molecular interaction between K103N+L100I and L74V mutations at the reverse transcriptase gene *in vivo*, limiting the future use of second-generation non-nucleoside reverse transcriptase inhibitors such as etravirine.

Introduction

Incomplete suppression of HIV type-1 (HIV-1) replication in infected patients undergoing antiretroviral (ARV) therapy is the main cause of viral resistance. The degree of resistance depends not only on the viral genetic barrier for drug resistance, defined as the number of nucleotide substitutions required to overcome drug-selective pressure, but also on the type and combination of mutations selected by the treatment regimens. When ARV resistance mutations appear in the HIV-1 genome, they can persist and affect the effectiveness of treatment, causing virological failure and further limiting future treatment options through cross-resistance.

Resistance to ARV drugs is mainly mediated by amino acid substitutions in the HIV-1 *pol* gene, specifically in protease (PR) and the first 220 codons of reverse transcriptase (RT). During therapy, the selection of combinations of mutations is mainly influenced by the effect of each mutant on viral fitness and replication capacity. Although some combination of amino acid changes at the *pol* gene decrease RT enzyme activity or impair viral replication [1], others confer an evolutionary advantage to HIV-1 mutants under ARV selection pressure [2]. Another factor conditioning HIV-1 resistance pathways is the viral genetic background. Non-B HIV-1 subtypes have shown to select for mutations on the *pol* gene that differ from those observed in subtype B viruses, possibly because of the differential effect of baseline polymorphism on the evolution of drug resistance along distinct mutational pathways [3–5].

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In Argentina, a variety of HIV-1 BF recombinants represent approximately 85% of the HIV-1 strains among the heterosexual and infant population [6,7] (PCA *et al.*, unpublished data). Previous studies of ARV resistance profiles in BF recombinant *pol* genomes from treatment-naive patients showed differences in baseline polymorphisms in relation to subtype B strains [8] and also in the frequency of ARV-associated mutations in treatment-experienced adult patients [9,10]. However, the prevalence and association of ARV resistance mutations in relation to treatment history – especially in children – and their effect on future treatment options has not been sufficiently unveiled in BF recombinants; therefore, our aim was to analyse the prevalence and association of drug resistance mutations and their relationship with treatment history in a group of long-term vertically infected patients from Argentina.

Methods

Patients

A total of 135 HIV-1 vertically infected patients had genotypic ARV resistance tests performed between February 2006 and October 2008 in our laboratory (Laboratorio de Biologia Celular y Retrovirus, Hospital de Pediatria 'JP Garrahan', Bueno Aires, Argentina) as part of routine follow-up. Of these, 71 patients (38 male and 33 female) had documented treatment failure to ≥2 ARV regimens. At the time of study, 38 were children (≤12 years old) and 33 were adolescents (between 13 and 21 years old).

HIV-1 plasma RNA copies were measured simultaneously with the genotypic ARV resistance test in 65 of the 71 (91.5%) samples using HIV-1 RNA Cobas® TaqMan 48 (Roche Diagnostic Systems, Branchburg, NJ, USA). $CD4^+$ T-cell counts were measured using flow cytometry (FACS Sorter; Becton Dickinson, San Jose, CA, USA) on whole blood samples within 3 months of the genotypic ARV resistance tests, and were also available in 66 of the 71 (92.9%) samples.

Clinical, demographical and virological information, as well as laboratory results, were obtained from the clinical records and informed consent was obtained from the parents or legal guardians in all cases.

HIV-1 amplification

HIV-1 RNA was extracted from 140 µl of plasma with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and 5 µl were reverse transcribed with random primers (Biodynamics, Buenos Aires, Argentina) in a final volume of 20 µl. To amplify a 1,168 base pair (bp) *pol* fragment (HXB2 coordinates 2,068–3,236 bp), two rounds of PCR were performed with the outer primers PRA (5′-CCTAGGAAAAAG GGCTGTTGGAAATGTGG-3′) and IBR1 (5′-AA CTTCTGTATATCATTGACAGTCCA-3′), and the inner primers PRB (5-′ACTGAGAGACAGGCTAA TTTTTTAGGGA-3′) and IBR2 (5-′CAAAGGAATG GAGGTTCTTTCTGATG-3′). DNA was amplified in 50 µl reaction mixtures containing 1× Taq Buffer, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 U Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 4 mM $MgCl_2$ or 1.5 mM $MgCl_2$ in the first and second rounds, respectively. Cycling conditions were 5 min at 95°C, 35 amplification cycles of 30 s, denaturation at 95°C, 30 s annealing at 55°C and 2 min 30 s extension at 72°C (first round) or 1 min extension at 72°C (second round) followed by soaking at 15°C. Second-round PCR products were purified with QIAquick columns (Qiagen) and semiquantified in 1.5% agarose gels against a pGEM marker (Promega, Madison, WI, USA) for downstream sequencing reactions.

HIV-1 genotyping and prediction of drug resistance

At least four overlapping forward and reverse sequencing reactions were carried out by the dideoxy method using the DYEnamic ET Terminator Cycle sequencing kit (Amersham Biosciences, Buckinghamshire, UK) to assemble the complete 1,168 bp *pol* fragment for each sample. Sequences were run in an ABI PRISM 310 Automated Sequencer (Applied Biosystems, Foster City, CA, USA), analysed with the DNA Sequencing Analysis software version 3.3 (Applied Biosystems) and assembled with the MEGA version 3.1 programme [11]. The Stanford University genotypic resistance interpretation algorithm (HIVdb programme version 4.3.6) was used on *pol* sequences to look for ARV drug resistance mutations and to predict the level of resistance to the available ARV drugs [12]. Drug resistance-associated mutations for protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) were identified in accordance with the 2008 recommendations of the International AIDS Society–USA [13]. The percentage of samples with resistance to each class of ARVs was calculated by submitting *pol* sequences to the calibrated population resistance (CPR) version 3.0 beta implemented at Stanford University HIV Drug Resistance Database [12]. Sequences were submitted to GenBank under accession numbers FJ525802 to FJ525872.

Subtyping and phylogenetic analyses

BF recombination breakpoints were analysed by bootscan analysis with Simplot version 2.5 [14] based on 100 resamplings, supporting branching with subtype B, F and A (outgroup) reference sequences within a 200 bp window moving in steps of 20 bases. Phylogenetic trees were constructed by neighbour-joining using the Tamura-Nei model, implemented in MEGA

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3.1 programme, and bootstrap analyses were done to assess the stability of the nodes.

Pairwise association between ARV resistance mutations

Associations between ARV resistance mutations were analysed independently for PR and RT by pairwise non-parametric tests. Each resistance-associated amino acid site was coded into a binary variable according to presence or absence of ARV resistance-conferring residues. Site-to-site pairwise associations were evaluated on 2×2 contingency tables by Fisher's exact two-tailed tests. To avoid an inflated type-I error rate (frequency of false-positive tests), two approaches were sequentially implemented: the number of tests was narrowed according to their *a priori* power under the alternative hypothesis and two corrections for multiple comparisons procedure were alternatively applied. The Bonferroni method was applied to control the per family error rate (the expected number of false-positive tests) and consequently to the familywise error rate (the probability of at least one false-positive test). The Benjamini and Hochberg procedure was followed to ensure an upper limit to the false discovery rate (the fraction of false-positives among all significant tests). The script (on R/S language) and *P*-values for all the statistically significant associations can be found in Additional file 1.

Association between ARV resistance mutations and treatment history

To identify significant associations between ARV resistance mutations and treatment regimens, we performed pairwise correlation analyses between each mutation and the administration of ARVs either in an historical time or at the time of the genotypic resistance testing as two independent variables on 2×2 contingency tables by Fisher's exact two-tailed tests as described earlier.

Results

Patient characteristics

All patients under study were HIV-1-infected by vertical transmission and had experienced treatment failure at the time of the genotypic ARV resistance test. The median time of infection was 12.8 years. Clinical characteristics and treatment history are summarized in Table 1. A high proportion (60.5%) of patients received monotherapy or dual therapy during the pre-highly active ARV therapy era (before 1996 in Argentina), with zidovudine (AZT), AZT+ didanosine (ddI) or stavudine (d4T)+ddI. As part of the ARV therapy, patients received a median of four different regimens that included NRTIs in all cases (70 lamivudine [3TC], 69 AZT, 63 ddI, 62 d4T, 41 abacavir

Table 1. Clinical characteristics and treatment history of HIV-1 vertically infected patients according to the antiretroviral mutation profile selected on the RT gene

Characteristic	Total (*n*=71)	With RT mutations L74V plus L100I+K103N (*n*=7)
Female gender, *n* (%)	33 (46.5)	4 (57.1)
Median time of HIV-1 infection, years (range)	12.8 (3.8–20.1)	13.2 (9.7–15.3)
Median HIV-1 RNA, $\log_{10}$ copies/ml (range)	4.9 (2.9–5.9)	4.7 (4.5–5.0)
Median $CD4^+$ T-cell count, cells/mm^3 (range)	415 (12–1,584)	640 (176–1,526)
Patients that received mono/dual therapy pre-HAART, *n* (%)	43 (60.5)	4 (57.1)
Time of mono/dual therapy pre-HAART, years (range)	1.6 (0.5–7.2)	2.3 (0.9–3.4)
Time of HAART therapy, years (range)	8.5 (2.3–10.7)	8.9 (6.7–10.3)
Median number of treatment regimens, *n* (range)	4 (2–8)	4 (2–7)
Drug used in the last treatment regimen		
Zidovudine, *n* (%)	12 (17)	1 (14)
Didanosine, *n* (%)	15 (21)	1 (14)
Stavudine, *n* (%)	34 (47.9)	1 (14)
Lamivudine, *n* (%)	17 (24)	3 (43)
Abacavir, *n* (%)	34 (47.9)[a]	6 (86)[a]
Tenofovir disoproxil fumarate, *n* (%)	13 (18.3)	3 (43)
Nevirapine, *n* (%)	5 (7)	0 (0)
Efavirenz, *n* (%)	28 (39.4)[a]	6 (86)[a]
Protease inhibitors	35 (49.3)	1 (14)
HIV subtype in *pol* fragment		
BF, *n* (%)	65 (91.5)	7 (100)
B, *n* (%)	6 (8.5)	0 (0)

[a]Statistically significant differences between groups with and without mutations L74V and L100I+K103N (Fisher's exact test *P*-value <0.05). HAART, highly active antiretroviral therapy; HIV-1, HIV type-1; RT, reverse transcriptase.

[ABC] and 14 tenofovir disoproxil fumarate), PIs in all cases (52 nelfinavir [NFV], 44 ritonavir [RTV], 18 lopinavir [LPV], 16 saquinavir [SQV], 16 indinavir [IDV], 8 fosamprenavir [APV], 3 atazanavir [ATZ] and 1 tipranavir [TPV]) and NNRTIs in 50 of the 71 patients (40 efavirenz [EFV] and 17 nevirapine [NVP]). All PIs (except for RTV and NFV) were boosted with RTV. Detailed information of the historical and the last ARVs administered to the patients, as well as the ARV resistance-associated mutations observed in the genotypic ARV resistance test can be found in Additional file 2.

At the time of study, the proportion of patients that developed resistance mutations to each class of ARVs was 98.6% for NRTIs, 83.1% for PIs and 90% for NNRTIs. Resistance mutations to NRTIs plus NNRTIs were observed in 63.4% of the HIV-1 isolates and triple-class resistance in 47.9%.

Prevalence and clinical association of HIV-1 resistance mutations in PR

Individual prevalence of PI resistance mutations was registered in subtype B and BF sequences, and shown in Figure 1A. The most frequent amino acid changes occurred at positions M36 (78.9%), L10 (70.4%), K20 (60.6%), L63 (57.7%), V82 (53.5%), I54 (49.3%), A71 (47.9%), I93 (43.7%), L90 (38.0%) and M46 (33.8%). Of these, only changes at positions 46, 54, 82 and 90 were defined as major mutations, whereas the others were either minor resistance mutations or non-subtype B polymorphisms, with little or no effect on ARV susceptibility *per se* [13].

PI resistance-associated mutations M46I/L, I54M/L/V/A/S and V82A/F/T/S/M/I were significantly associated with each other and occurred in a relatively high proportion of cases (Figure 1B; V82+I54 [45.1%], M46+V82 [29.6%] and M46+I54 [26.8%]). Major PI mutations at codons 54 and 82 were also associated with minor PI mutations at positions 10, 24 and 71. Also, mutations at some pairs of polymorphic sites were found to be associated (K20+M36 [60.6%], M36+L63 [54.9%] and L63+I93 [36.6%]) in this and other ARV-experienced cohorts [9,10], and also in BF HIV-1 genotypes from drug-naive patients from Argentina [8,15] (PCA, unpublished data), reflecting the local genetic variability of HIV-1 strains. Another association was found between mutations D30N+N88D (15.5%), reflecting one of NFV resistance pathways.

When we examined the correlation between PI-based treatment regimens and mutations at PR, only one statistically significant association occurred between mutations V82A/F/T/S/M/I and the administration of RTV in an historical treatment regimen ($P<0.05$). Interestingly, RTV was the PI most frequently used in this population (Additional file 2) at least until the year 2000, when it was replaced by more potent PIs boosted with RTV, favouring the persistence of RTV-selected mutations over time.

Prevalence and clinical association of HIV-1 resistance mutations in RT

At the RT gene, the most prevalent amino acid changes associated with NRTI resistance were M184I/V (60.6%), M41L (53.5%), D67E/T/N (50.7%), T215Y (50.7%) and T69A/D/E/G/I/K/N/S (42.2%), whereas the most frequent mutations conferring NNRTI resistance were K103N/S (33.8%) and Y181C/I/V (19.7%; Figure 2A).

Of the eight pairs of mutations associated with statistical significance at RT (Figure 2B), three conform to the thymidine analogue mutation profile 1 (TAM)1 [16]: M41L+L210W (26.8%), L210W+T215Y (25.3%) and M41L+T215Y (45.1%). Mutations, K70R/D/T and K219Q/E (18.3%), belonging to the TAM2 profile were also significantly associated. The TAM1 profile was more common (18 patients) than TAM2 profile (13 patients).

Correlations between mutations at RT and treatment regimens included M184V/I and 3TC or ddI in the last treatment regimen; K103N/S and EFV in an historical or last treatment regimen; L100I and EFV in the last treatment regimen; and Y181C/I/V and NVP in an historical regimen.

Of the NNRTI resistance mutations, the compensatory mutation L100I showed a strong association with K103N/S, occurring in 15.5% of the cases, and also with the NRTI resistance mutations L74V/I in 11.3% (Figure 2B).

Association between K103N+L100I and L74V mutations at the RT gene

Of the 50 patients who received NNRTIs, 45 carried NNRTI resistance mutations. Of these, 23 (51.1%) selected the K103N resistance mutation, which was associated with the L100I minor mutation in 11 (47.8%) patients. K103N+L100I was the most common combination of NNRTI resistance mutations in our study group. The statistical association found between K103N/S and L100I was maintained for K103N+L100I as only one sequence carried a serine in RT at codon 103 and this was not in association with L100I. Most of the patients with K103N+L100I also harboured the NRTI-associated resistance mutation L74V (7/11; Fisher's exact test $P<0.001$). Another patient carried K103N+L100I in combination with the L74I mutation, but this patient was excluded from further analyses as the L74V and L74I mutations seemed to correspond to different mutational pathways [17]. To identify the possible causes for the association of K103N+L100I and L74V mutations, we examined treatment history of patients with or without the mutations (Table 1). As expected, the association of K103N+L100I and L74V was significantly more frequent in patients receiving

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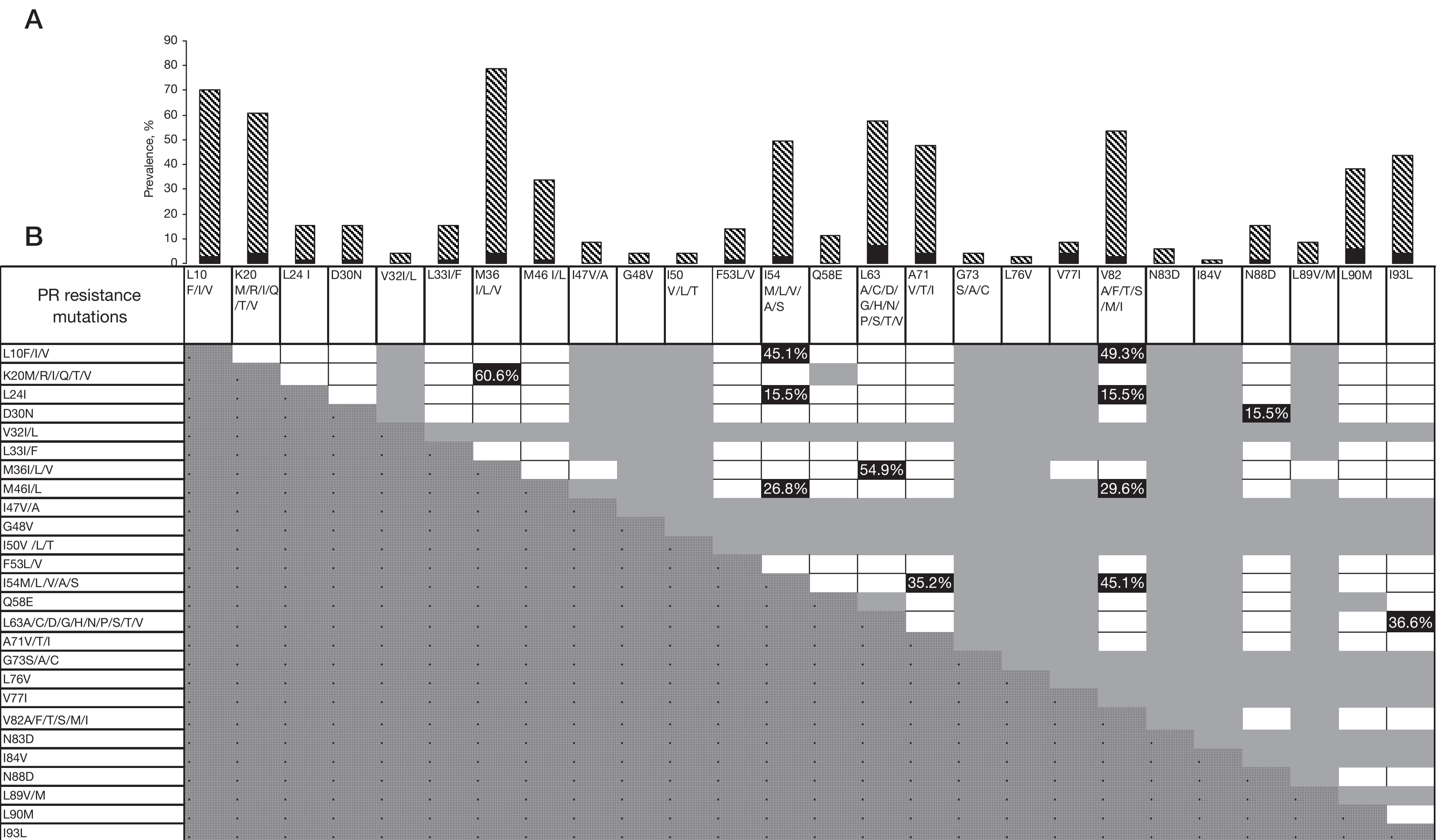
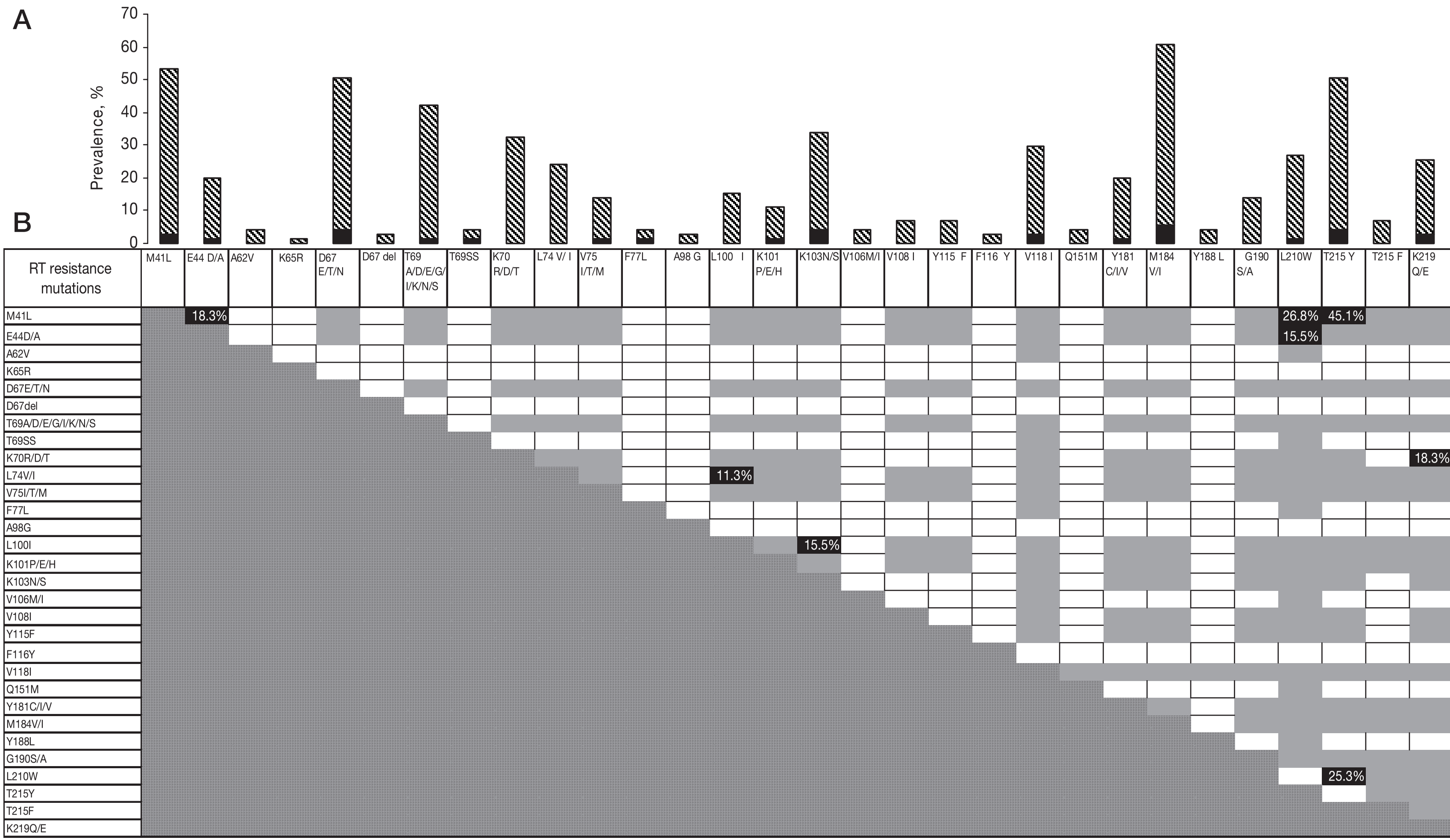
Figure 1. Frequency and association of PI-associated resistance mutations



(A) Bar graph representing the frequency of protease inhibitor (PI)-associated resistance mutations in the study group (n=71). The x-axis represents each of the amino acid changes considered to contribute to PI resistance and the y-axis shows the cumulative frequency observed in subtype B (black bars; n=6) and BF recombinant (striped bars; n=65) sequences. (B) Contingency tables with pairwise associations for all PI-associated resistance mutations considered. The upper right half of the table was used to show the results: squares shaded in grey indicate associations not tested because the frequency of one or both mutations was insufficient to perform the Fisher's exact test with sufficient power. Blank squares indicate associations tested but with no statistical significance (P>0.05) and black squares show statistically significant (P<0.05) associations. The frequency of the combined mutations is shown inside the black squares. PR, protease.

Figure 2. Frequency and association of NRTI- and NNRTI-associated resistance mutations



(A) Bar graph representing the frequency of nucleoside reverse transcriptase inhibitor (NRTI)- and non-nucleoside reverse transcriptase inhibitor (NNRTI)-associated resistance mutations in the study group (n=71). The x-axis represents each of the amino acid changes considered to contribute to viral resistance and the y-axis shows the cumulative frequency observed in subtype B (black bars; n=6) or BF recombinant (striped bars; n=65) sequences. **(B)** Contingency tables with pairwise associations for all reverse transcriptase (RT) inhibitor-associated resistance mutations considered. The upper right half of the table was used to show the results: squares shaded in grey indicate associations not tested because the frequency of one or both mutations was insufficient to perform the Fisher's exact test with sufficient power. Blank squares indicate associations tested but with no statistical significance (P>0.05) and black squares show statistically significant (P<0.05) associations. The frequency of the combined mutations is shown inside the black squares.

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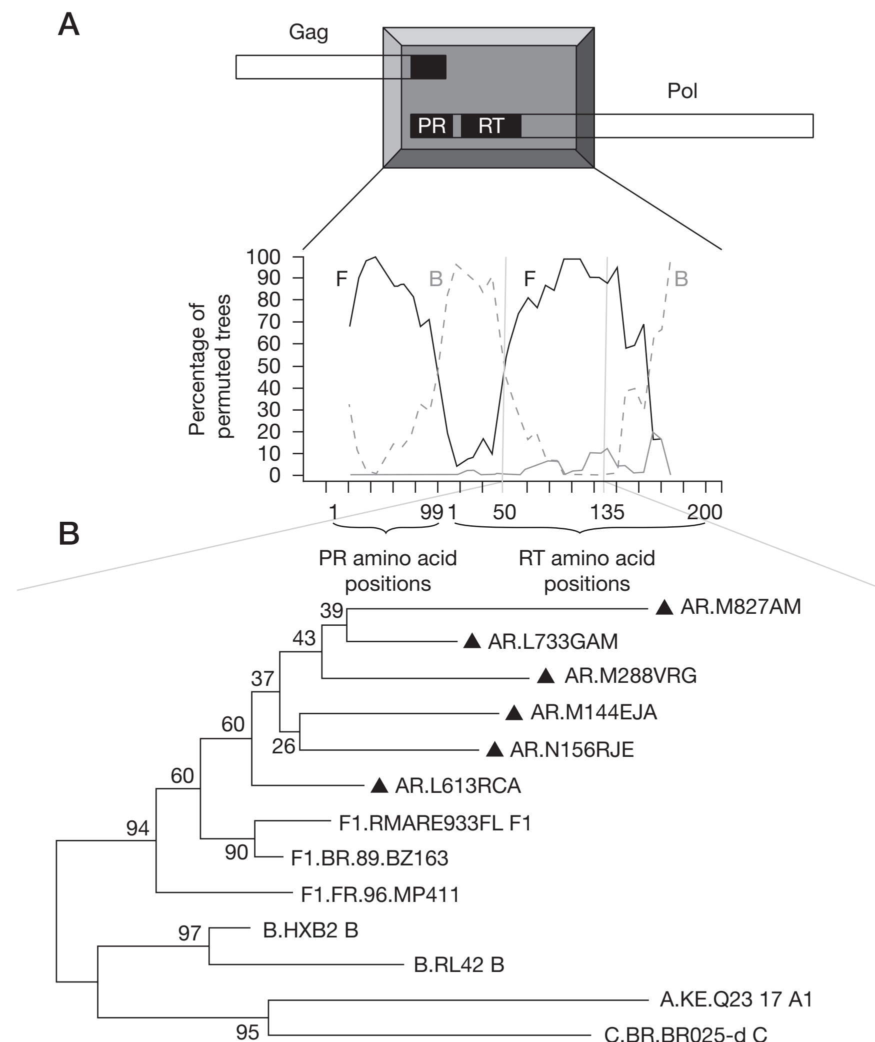
EFV (86% versus 34%) and/or ABC (86% versus 44%) in their last treatment regimen (Fisher's exact test $P<0.05$), but was independent of the time under any of these ARVs (PCA, data not shown). Because L74V has been previously documented to be strongly linked to ddI [17], an association with this NRTI would also be expected for patients carrying triple mutants; however, we did not find a significant association between the presence of L100I+K103N and L74V mutations, and on-therapy treatment with ddI, or with prior experience with suboptimal (single or double) nucleoside regimens (Table 1). In the latter case, the selection of L74V mutation could be expected to occur and remain as a background mutation, thereby allowing the further selection of K103N+L100I, but we could not confirm this hypothesis. Interestingly, we observed that K103N+L100I plus L74V mutations were exclusively present in BF recombinant *pol* genomes and were absent in all the subtype B strains.

HIV-1 *pol* subtype

Through phylogenetic and recombination analysis of the HIV-1 *pol* fragment spanning the 99 PR codons and the first 220 codons of RT, we identified BF recombinant

Figure 3. HIV type-1 subtype characterization in *pol*



(A) Representative bootscanning plot of HIV type-1 *pol* sequences from the six of the seven isolates sharing L74V, L100I and K103N mutations, depicting a BF mosaic structure with a common subtype F fragment between codons 50 and 135 of reverse transcriptase (RT). Black lines represent subtype F, grey dashes represent subtype B and grey lines at the baseline represent subtype A (used as outgroup). **(B)** Neighbour-joining phylogenetic analysis of nucleotide sequences spanning codons 50–135 of RT. The numbers near the nodes are the percentage of bootstrap replicates supporting the clades and the scale indicates substitutions per site and refers to the horizontal branch lengths. Subtype B, F, C and A references were used. Full triangles indicate samples sharing L74V, L100I and K103N mutations in the RT gene. PR, protease.

HIV-1 genomes in 65 of 71 (91.5%) patients and subtype B viruses in 6 (8.5%) patients.

All seven HIV-1 isolates with L74V, L100I and K103N mutations on the RT gene were BF recombinants and six of them shared a similar BF mosaic pattern by bootscan recombination analysis (Figure 3A). In all these isolates, the three mutations were included in a subtype F segment that comprised RT codons 50–135 (Figure 3B). Only one *pol* sequence carrying L74V, L100I and K103N mutations had a different mosaic structure, with a shorter subtype F fragment starting at RT codon 96 and ending at RT codon 141.

Discussion

In the present work, we analysed the prevalence and association of drug resistance mutations of the HIV-1 PR and RT genes in a group of long-term vertically infected patients from Argentina who were mostly infected with BF recombinant HIV-1 strains by means of a comprehensive statistical approach.

A high prevalence of drug resistance mutations was observed for the three classes of ARV drugs in the study group. Major PI mutations in the PR gene at positions 46, 54 and 82 showed a strong statistical association between each other and changes at position 82 were also strongly associated with the use of RTV in a historical treatment regimen. Our results partially agree with those previously reported, where mutations at codon 82 were highly associated with K20R/M and I54V/L in BF recombinant strains from adult patients from Argentina (74% versus 19% in subtype B; $P<0.001$) [18]. We hypothesize that at least two factors led to the emergence and persistence of V82 RTV-selected mutants (either alone or in combination with other mutations) in the study population: the frequent use of non-suppressive exposure to RTV at an early time and its sustained use at minimal doses in subsequent PI-based treatment regiments, and convergent mechanisms by which the same mutations are selected by the virus under exposure to different PIs. Whether the association of L10 polymorphisms and major mutations at PR position 54 or 82 has any effect on facilitating the selection or persistence of these mutants is a matter that will need to be further explored. In clinical practice, these patients will have limited options for treatment with PIs, as a moderate to high resistance to LPV, IDV, NFV, ATZ and APV will be expected, although the chances of using darunavir, SQV or TPV will depend on the combination of all the mutations present in the viral PR.

In the RT gene, K103N/S were associated with L100I under treatment with EFV. Although the mutation L100I was quite frequent in our clinical dataset (15.5%), it was either not reported or found at a low percentage in adult populations from Argentina [9,10]. In other clinical settings, K103N+L100I double mutants were observed at low prevalence (<10%) and other NNRTI resistance mutations seemed to be preferred in combination with K103N/S [19,20].

Interestingly, the frequency of K103N in combination with L100I seemed to increase when the L74V mutation was also present. For example, a high prevalence of K103N+L100I (37%) was described in a group of ARV-experienced patients receiving an EFV/ABC/APV salvage regimen [21] and also in a group of 574 NRTI+NNRTI experienced patients carrying subtype B strains from an Italian cohort [22]. In agreement with our results, an association between L74V and L100I was observed in these reports, although it only reached statistical significance in the latter.

Koval *et al.* [23] suggested that the lower than the expected frequency of K103N+L100I double mutants in clinical settings could be the result of a fitness impairment in the viral population carrying the mutations, which was compensated by the L74V NRTI resistance mutation. This beneficial interaction seemed to be independent of the effect of L74V on NRTI resistance [24,25]. Moreover, L74V showed a 5.6-fold increase in a large group of subtype B HIV-1-infected patients failing NRTIs+NNRTIs in comparison to those failing only NRTIs [22], indicating an effect on NNRTI resistance that was independent of the patients' experience with ddI or ABC. By means of a statistical approach that takes into account the large number of possible comparisons, we confirmed the association between RT mutations L74V, L100I and K103N, indicating that the molecular interaction between these mutations also occurs in BF recombinant strains. The preferential selection of K103N+L100I in the context of L74V mutation in our study group was, in part, related to the administration of EFV and ABC during the ongoing treatment regimen at the time of genotypic resistance testing; however, other factors, such as HIV-1 subtype, might also have contributed to the results obtained as triple mutants carrying K103N+L100I and L74V mutations were only concomitantly found in a subtype F fragment within the BF recombinant *pol* gene. Although differences in the frequency of ARV resistance mutations among HIV-1 subtypes are not unusual [5] and were even reported for K103N [26], a further number of B and BF viral genotypes will need to be studied in order to determine the real impact of viral subtype on the selection of K103N+L100I and L74V mutations.

The high number of L100I+K103N double mutants observed in our group of long-term vertically infected patients is clinically relevant because of the expanded resistance to second-generation NNRTIs such as etravirine (ETV), an ARV of choice in patients with multiple treatment failures. In the DUET-1 and -2 studies, L100I was identified as 1 of the 13 mutations associated with

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decreased virological response to the new drug ETV and reported a fold change of >10 for ETV for the combination of mutations L100I+K103N [27]. By weighting the contribution of each NNRTI resistance mutation according to the ETV resistance score assembled by Vingerhoets *et al.* [28], 2.8% of our patients had a reduced response to ETV (score ≥4), whereas another 19.7% had intermediate response to this new drug (score between 3.5 and 2.5). The high prevalence of ETV resistance in our group of patients is concerning.

In conclusion, trends in drug usage have changed over the years since the introduction of AZT as the first ARV in 1996, shaping the patterns and pathways of drug resistance-associated mutations in the HIV-1 genome. By doing so, the NRTI resistance profile in our group included the mutations of the TAM1 complex (M41L+L210W+T215Y) and the ddI/3TC-associated mutations M184V/I. For NNRTIs, the most common resistance mutations were Y181C/I/V and K103N/S, and an association was observed between NNRTI (K103N+L100I) double mutants, and the NRTI-associated mutation L74V. Our results could be explained by a fitness advantage of viruses carrying these mutations over other possible combinations. In the PR gene, PI resistance profiles reflect the intrinsic genetic variability of BF recombinants, and the frequent use of RTV as the first PI in our group of patients, followed by other PIs in more recent ARV treatment regimens.

Without enough ARV effect to achieve viral load suppression, resistance will emerge, limiting future options. Identifying the ARV resistance pathways generally preferred in non-B HIV-1 subtypes is crucial for the selection of adequate and effective treatment regimens in individuals with long-term infection.

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Disclosure statement

The authors declare no competing interests.

Additional files

Additional file 1: A detailed description and the R/S language script for the multiple comparison procedure, as well as the statistically significant pairwise associations between ARV mutations can be found at http://www.intmedpress.com/uploads/documents/AVT-09-OA-1410_Aulicino_Add_file1.pdf

Additional file 2: Detailed information of the historical and last ARVs administered to the patients, as well as the ARV resistance-associated mutations observed in the genotypic ARV resistance test can be found at http://www.intmedpress.com/uploads/documents/AVT-09-OA-1410_Aulicino_Add_file2.pdf

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