Virology 456-457 (2014) 310–318

Contents lists available at ScienceDirect

Virology

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

Horizontal gene transfer from human host to HIV-1 reverse transcriptase confers drug resistance and partly compensates for replication deficits

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ARTICLE INFO

Article history:
Received 20 December 2013
Returned to author for revisions
24 January 2014
Accepted 22 March 2014
Available online 25 April 2014

Keywords:
HIV/AIDS
Antiretroviral therapy
Resistance
Insertion
Reverse transcriptase

ABSTRACT

We investigated the origin and the effect of insertion D67D-THGERDLGPA within HIV-1 RT from a patient failing antiviral therapy. The insertion developed within the context of pre-existing NRTI and NNRTI mutations (M41L, L210W, T215Y and N348I). Concurrently, the NRTI mutations T69I and V118I and the NNRTI mutations K103N and Y181C were detected for the first time. High-level drug resistance (fold-changes ≥ 50) and a good replication capacity (87% of wild-type) were observed, significantly higher than for the previous virus without insertion. The insertion was very similar to a region within human chromosome 17 (31/34 nucleotide identity), and had already been detected independently in a Japanese HIV-1 isolate. These results suggest that a particular sequence within human chromosome 17 is prone to horizontal gene transfer into the HIV-1 RT finger subdomain. This insertion confers selective advantage to HIV-1 by its contribution to multi-drug resistance and restoration of impaired replication capacity.

Introduction

Nucleoside reverse transcriptase (RT) inhibitors (NRTIs) still remain the cornerstone of current antiretroviral therapy (ART) against human immunodeficiency virus type 1 (HIV-1) (DHHS, 2014). Initial treatment regimens usually consist of 2 NRTIs in combination with either 1 non-NRTI (NNRTI), 1 boosted protease inhibitor (PI/r) or 1 integrase inhibitor (INI). Even in second line regimens, NRTIs form the backbone of the combination ART (cART), provided NRTI resistance is not too extensive. Moreover, NRTIs are important players in the prevention of mother to child transmission and their role in treatment as prevention (TasP) and pre-exposure prophylaxis (PrEP) is being extensively investigated (Gupta et al., 2013). During the last few years the gamut of antiviral drugs available for the treatment of HIV-1 has increased and diversified substantially. This has contributed to the successful decrease in morbidity and mortality in HIV-1 patients, even in patients who had failed multiple antiretroviral regimens (Gulick et al., 2008; Lazzarin et al., 2003; Madruga et al., 2007; Steigbigel et al., 2008).

Due to the high replication rate, the lack of proofreading activity and the template switching of RT, drug-resistant genetic variants can develop which may limit the success of cART. In this respect, HIV-1 can develop resistance against all NRTIs, either through mutations that selectively discriminate between the inhibitor and the dNTP or through mutations that enhance the excision of the incorporated inhibitor (Menendez-Arias, 2010). For example, the thymidine analog mutations (TAMs) are a combination of M41L, D67N, K70R, L210W, T215Y/F and K219Q/E

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http://dx.doi.org/10.1016/j.virol.2014.03.023
0042-6822/

in RT, mainly selected by thymidine analogs zidovudine (AZT) and stavudine (d4T), but conferring resistance to all NRTIs when multiple mutations are present (Demeter et al., 1995; Kellam et al., 1992; Larder and Kemp, 1989). Another form of multi-NRTI resistance is referred to as the Q151M pathway (A62V, V75I, F77L, F116Y and Q151M) (Shafer et al., 1994). High-level resistance to antiviral drugs generally arises by stepwise accumulation of mutations, rather than insertions or deletions (Johnson et al., 2013), but insertions and deletions contributing significantly to drug resistance have been described. These seem especially important in affecting the RT finger subdomain and have occasionally been observed in HIV-1 patients not responding to cART with a prevalence ranging from 0.6% to 4.1% (Andreoletti et al., 2002; Balotta et al., 2000; Briones et al., 2001; Bulgheroni et al., 2004; de Jong et al., 1999; Kaliki et al., 2000; Larder et al., 1999; Lukashov et al., 2001; Mas et al., 2000; Masquelier et al., 2001; Rakik et al., 1999; Ross et al., 1999, 2000; Sato et al., 2001; Schneider et al., 2004; Sugiura et al., 1999; Tamalet et al., 1998, 2000; van der Hoek et al., 2005; Van Vaerenbergh et al., 2000; Winters et al., 1998).

The aim of this study was to investigate the origin of an uncommon 10-amino acid insertion within the finger subdomain of HIV-1 RT from a patient failing cART. We want to address the impact of the insertion by characterizing the genotypic and phenotypic viral profile.

Results

Therapy history and in vivo evolution of genotypic drug resistance

The Belgian HIV-1 patient was diagnosed in 1993 with a subtype A strain and was presumably heterosexually infected in Sub-Saharan Africa. He received AZT monotherapy for a short period in 1994 (Fig. 1). The surveillance began in 1996, but a baseline plasma sample was absent. However, two PBMC samples isolated while the patient was receiving AZT and zalcitabine (ddC) were available for retrospective drug resistance analysis. Genotypic drug resistance testing was performed on plasma samples as part of routine clinical care between 2000 and 2007. Sequence analysis revealed the presence of M41M/L, L210L/W and T215T/N/Y/S (sample 1), followed by the full replacement of M41L and T215Y, together with the disappearance of L210W (sample 2). Both samples contained the mutations K20K/R and L89M in protease. In 1997, AZT and ddC were replaced by 3TC and d4T. A few months later, saquinavir mesylate (SQV) was added to the regimen, subsequently replaced by indinavir (IDV) after a few more months. At the beginning of 1999, boosted saquinavir (SQV/r) was initiated instead of IDV and ABC replaced 3TC. During treatment with this regimen, the NRTI mutation L210W and the NRTI/NNRTI mutation N348I appeared together with the PI mutation I84V and the full replacement of K20R (sample 3). Under the selective pressure of IDV/r, ABC and NVP, sample 4 was isolated in which the insertion D67D-THGERDLGPA and the mutations T69I, K103N, V118I and Y181C were also observed within RT. However, the NNRTI mutation N348I and the PI mutation I84V had disappeared. Subsequently, the NRTI mutations L74L/I and T215C, the NNRTI mutations K101K/Q and G190G/A, and the PI mutations K20R/I, I54V, V82A and L89M/I appeared under selective pressure of ABC, EFV and boosted lopinavir (LPV/r) (sample 5). Moreover, V118I was absent at this time. On the patient's final regimen (ddI, ABC, fosamprenavir (FPV) and LPV/r), K20R in PR and M41L and L74I in RT were not observed anymore, whereas A98A/G, K103N/H, V118V/I and G190A were also detected in RT and L10F and T74A in PR (sample 6).

in vivo evolution of insertion

PCR fragments containing the RT gene of the last clinical sample without insertion (sample 3) and the first with insertion (sample 4) were cloned. Nine clones from each sample were sequenced. None of the clones from sample 3 displayed the insertion, in contrast to the clones from sample 4 that all contained the insertion (Fig. 2). The insertion at RT position 67 remained present during the entire investigated time period of 6 years, although some residues did evolve. In sample 4, one clone displayed arginine (Arg) at the second amino acid position within the insertion. For the last 2 samples, only population-based sequences were obtained. Sample 5 displayed a mixture of histidine (His) and tyrosine (Tyr) at the second amino acid position in the insertion. Mixtures of threonine (Thr) and proline (Pro) at the first and of leucine (Leu) and glutamine (Gln) at the seventh amino acid position of the insertion were present in sample 6.

Drug susceptibility and replication capacity

Clonal fragments representative of samples 3 and 4 were used to generate RT-recombinant viruses (Table 1). Sequencing of the recombinant viruses confirmed a genotypic profile within RT identical to the original clone. Drug susceptibility testing of sample 3 revealed resistance towards all tested NRTIs and NNRTIs, although not all values were significantly different from wild-type due to inter-assay variability (Table 1). The drug resistance levels varied for the NRTI drug class between 3-fold towards ddI and 267-fold towards AZT, and for the NNRTI drug class between 12-fold towards EFV and 83-fold towards NVP. From sample 4 it seems that drug resistance levels to all drugs increased substantially, with drug resistance levels ranging from 50-fold towards 3TC to more than 2130-fold towards AZT. Resistance against NVP increased to more than 168-fold and against EFV to 71-fold. Both viruses remained susceptible to PFA. The replication capacity of sample 3 was strongly impaired, reaching 34% of the HIV-1 wild-type's capacity (k-value 0.74 vs. 2.15). The capacity to replicate was partly restored in sample 4 which displayed 87% compared to HIV-1 wild-type (k-value 1.87 vs. 2.15).

Homologous nucleotide sequences to the observed insertion

As the insertion did not result from any duplication of nearby nucleotides, several PCR fragments covering the entire HIV-1 genome (sample 4) were sequenced to determine the origin of the 10-amino acid insertion. The BLAST program was used to compare its encoding nucleotide sequence plus 3 extra nucleotides adjacent to each side with the full HIV-1 genome. The highest scores were from two hits that consisted of 8/8 identities with an E-value of 2.4. As this analysis did not reveal an autologous origin of the entire insertion, all sequences available at the NCBI database were checked. This search revealed sequence homology with a region within human chromosome 17 (31/34 nucleotide identity, E-value of 0.002) and with the amino terminal part of RT from an HIV-1 isolate (99JP-NH3-II, 26/27 nucleotide identity, E-value of 0.006) (Fig. 2). All other hits, not related to the previous two, displayed E-values above 6.7.

Interestingly, the former hit was specifically matched with the reverse complement of a particular gene within human chromosome 17 (GenBank AC135178, NT positions 136,586–136,553) that encodes component 1 of the CTS telomere maintenance complex (GenBank NG032148, cytogenetic location 17p13.1, molecular location NT positions 8,128,138–8,151,412 of chromosome 17 (Genbank NC_000017.10)). We then sequenced a 339-bp PBMC DNA fragment to confirm that the patient-derived sequence was identical to AC135178 in GenBank. The match overlapped with an exon–intron boundary of the respective gene (NT positions 5099–5066).

The latter hit relates to a nucleotide sequence that encodes an 11-amino acid insertion at RT position 67 in an HIV-1 CRF01_AE isolate from a Japanese patient (GenBank AB053002)

(Sato et al., 2001). The insertion differed from the one observed in our study at 5 nucleotide positions and contained an extra codon (Fig. 2).

Genetic linkage between HIV-1 patients displaying a similar insertion

To ascertain whether the HIV-1 strains of the two patients were linked, we determined the phylogenetic relationships of the sequences. Our analysis included HIV-1 subtype A and CRF01_AE sequences from the local HIV-1 epidemics and the sequences most similar to the investigated strains according to BLAST. The strains from the Belgian and Japanese patient did not cluster together in the phylogenetic tree (Fig. 3). They belonged to the subtype A and CRF01_AE clusters, respectively, supported by bootstrap values of 94%. We can therefore exclude the possibility that this insertion occurred only once and was passed on between the two patients, and conclude that the insertion resulted from convergent evolution.

Mapping of the wild-type residues on HIV-1 reverse transcriptase

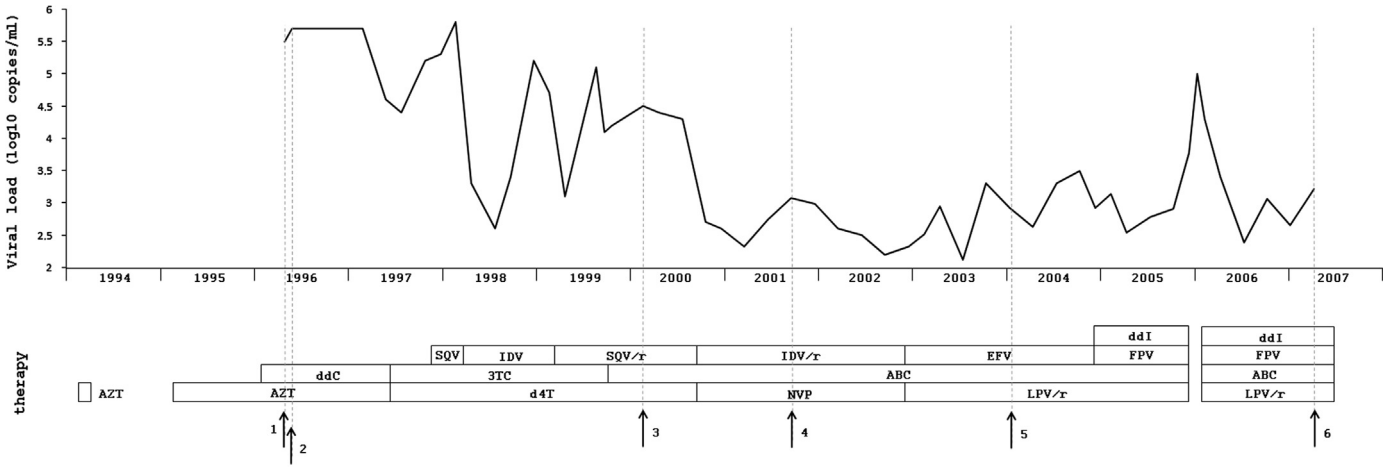
To provide structural evidence, the mutated residues and the insertion position were mapped to the crystalized RT in the presence of AZT and NVP (Fig. 4). This model confirmed the drug resistance conferred by the mutations and particularly by the insertion. The insertion, colored in red, was found to be near the catalytic area of RT where the dsDNA is synthesized, thereby impeding the binding of AZT. Furthermore, selected NNRTI mutations at positions 103 and 181 have an Euclidian distance of 4.95 Å and 4.66 Å to NVP, respectively, indicating strong contact at molecular level. Similarly, the selected NRTI mutations at positions 67–69 and 215 are close to AZT (Euclidean distances <8 Å). The positions 41, 118, 210 and 348 are located further away from those drugs, so a direct interaction with AZT and NVP is less likely.

Discussion

In an HIV-1-infected patient failing ART, a 10-amino acid insertion (THGERDLGPA) was observed at position 67 in the β3–β4 loop of the HIV-1 RT finger subdomain. The positions in RT most prone to insertions are located between RT codons 68 and 70. Insertions of two amino acids have mainly been reported at RT position 69 in clinical isolates from HIV-1 patients failing ART. These insertions typically confer NRTI resistance in association with TAMs (Larder et al., 1999; Mas et al., 2000; Meyer et al., 2003; White et al., 2004; Winters et al., 1998). As the finger subdomain traps the dNTP and precisely aligns the α-phosphate of the dNTP and the 3′-OH of the primer inside the polymerase active site, we wanted to investigate the origin and phenotypic effect of this large insertion near the catalytic site (Fig. 4).

Thymidine analogs seem to be important players in the selection process of 69-insertions as all but one study reported AZT therapy prior to their selection. In that study, two patients were exposed only to d4T (Ross et al., 1999). In patients developing 69-insertions, the most common course of treatment is AZT monotherapy, followed by a combination of AZT with either ddI or ddC. In the study patient, the insertion was only detected in samples after the combination of ABC+NVP+IDV/r was prescribed (Fig. 1). However, the patient had been pre-exposed to AZT monotherapy and several regimens containing either AZT+ddC, d4T+3TC and d4T+ABC.

The key role of thymidine analogs is also manifested by the occurrence of 69-insertions mainly within the context of TAMs, and more specifically within the TAM1 pathway (M41L, L210W and T215Y) (Larder et al., 1999; Matamoros et al., 2004; Scherrer et al., 2012; Tamalet et al., 2000). Although 69-insertions are typically preceded by a mutation from threonine to serine at position 69 (T69S) (Larder et al., 1999), our study described an insertion positioned between RT codons 67 and 68, which appears to have developed together with the mutation T69I. In the study patient, the TAM1 mutations were already detected in a sample from many years before the sample with insertion, whereas the minor NRTI



drug resistance mutations

sample	protease							reverse transcriptase												
	L10	K20	I54	T74	V82	I84	L89	M41	D67	T69	L74	A98	K101	K103	V118	Y181	G190	L210	T215	N348
1	-	K/R	-	-	-	-	M	M/L	-	-	-	-	-	-	-	-	-	L/W	T/N/Y/S	n/a
2	-	K/R	-	-	-	-	M	L	-	-	-	-	-	-	-	-	-	-	Y	n/a
3	-	R	-	-	-	V	M	L	-	-	-	-	-	-	-	-	-	W	Y	I
4	-	R	-	-	-	-	M	L	ins	I	-	-	-	N	I	C	-	W	Y	-
5	-	R/I	V	-	A	-	M/I	L	ins	I	L/I	-	K/Q	N	-	C	G/A	W	C	n/a
6	F	I	V	A	A	-	M/I	-	ins	I	-	A/G	K/Q	N/H	V/I	C	A	W	C	n/a

Fig. 1. History of viral load, therapy and drug resistance mutations in PR and RT. Only positions that are implemented within genotypic drug resistance interpretation systems Rega v8.0.2 or HIVdb v6.2 are enlisted. Positions associated with NNRTI resistance are underlined. n/a, not available. Positions according to NL4.3 (AF324493). 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; EFV, efavirenz; IDV, indinavir; IDV/r, ritonavir-boosted indinavir; FPV, fosamprenavir; LPV/r, ritonavir-boosted lopinavir; NVP, nevirapine; SQV, saquinavir; SQV/r, ritonavir-boosted saquinavir.

mutation V118I and the major NNRTI mutations K103N and Y181C were detected for the first time in the same sample as the insertion (Fig. 1).

It has been reported that the majority of 69-insertions do not confer resistance by themselves (Harrigan et al., 2007; Larder et al., 1999). In contrast to the most common insertion 69S-SS, the insertions 69S-SA and 69S-SG were nevertheless associated with some reduced NRTI susceptibility in the absence of other genotypic changes (Meyer et al., 2003). It is proposed that insertions and deletions within the β3–β4 HIV-1 RT loop increase the NRTI excision due to the destabilization of the dead-end complex instead of an increased ATP-binding (Boyer et al., 2001; Tu et al., 2010). In our study, the sample without insertion already displayed some NRTI resistance due to the TAM1 mutations and the level of resistance was strongly enhanced after the addition of the insertion (Table 1). Surprisingly, NNRTI resistance was observed in the absence of major NNRTI mutations and the level of resistance was further elevated after the selection of K103N and Y181C. The unexpected phenotypic NNRTI resistance prior to *in vivo* NNRTI exposure and without major NNRTI mutations could be explained by the presence of N348I. N348I is a mutation within the HIV-1 RT connection subdomain that previously had been reported in therapy-naïve and treated patients (0.8% and 12.1% respectively) (Yap et al., 2007). In patients treated with solely NRTI or with regimens containing NRTI and NNRTI, the mutation is usually observed in combination with TAMs with or without K103N/Y181C. These particular mutation combinations confer subsequently dual-class resistance, irrespective of the presence of major NNRTI mutations (Yap et al., 2007).

Regarding the impact of 69-insertions on the viral replication capacity, contradictory results have been reported. In the absence of drugs, insertions of different lengths had a negative impact on the replication capacities, although some studies reported replication capacities and polymerase activities comparable to wild-type (Huigen et al., 2007; Kew et al., 1998; Prado et al., 2004; Quinones-Mateu et al., 2002). In our sample, the insertion was able to partly restore the diminished replication capacity of the mutant virus displaying TAMs. Additionally, *in vivo* data suggested that the large insertion conferred selective advantage and was well tolerated as the insertion was retained for more than 6 years during a period in which cART was frequently changed.

Most of the reported HIV-1 insertions are simple duplications of neighboring sequences but a long-distance ectopic duplication of 15 nucleotides from the *env* into the *pol* gene has also been observed in HIV-1 (Lobato et al., 2002). However, the insertion present in the study patient was not a duplication of neighboring nor ectopic sequences. A BLAST search of all the sequences in GenBank revealed homology between our insertion and the

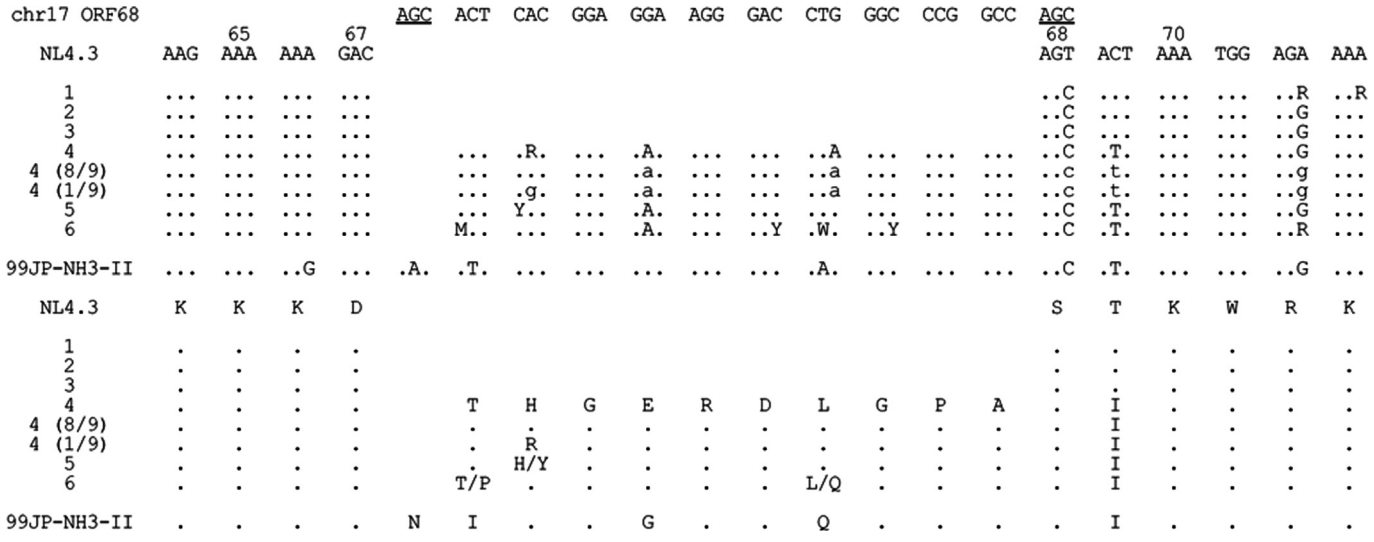


Fig. 2. Analysis of HIV-1 RT subdomain sequences. The upper and lower panels display respectively the nucleotide and amino acid alignment of the region immediately up- and downstream of the insertion. Positions are in accordance to HIV-1 NL4.3 (AF324493). The upper row is the reverse complement of the sequence fragment from chromosome 17 (open reading frame 68, chr17 ORF68) (AC135178) that matches to the insertion. 99JP-NH3-II is a clinical HIV-1 isolate from a Japanese child displaying a similar insertion (AB053002). Information on the 9 clones obtained for sample 4 is displayed in lower-case. Residues identical to the reference, whether NL4.3 or chr17 ORF68, are replaced by dots.

Table 1
Phenotypic characteristics of recombinant viruses.

Sample	Drug susceptibility[a]									Replication capacity [b]	
	Drug	AZT	d4T	ddI	3TC	ABC	TDF	NVP	EFV	PFA		
3	mFC ± SD	267 ± 76	6.36 ± 0.99	2.61 ± 0.31	3.8 ± 1.3	5.4 ± 2.6	13.0 ± 3.7	83 ± 34	12.4 ± 2.1	0.33 ± 0.12	*k*	0.74
	P-value	0.026	0.011	0.012	0.068	0.10	0.031	0.18	0.082	0.002	R^2	0.94
4	mFC ± SD	> 2130	> 84	52.8 ± 8.8	49.6 ± 8.7	> 263	54.4 ± 4.2	> 168	71.4 ± 4.7	0.50	*k*	1.87
	P-value	–	–	0.076	0.081	–	0.035	–	0.0015	0.21	R^2	0.99
3 vs. 4	*P*-value	–	–	0.0017	0.0023	–	0.0013	–	0.00050	0.28	*P*-value	0.012

[a] For each independent experiment, the mean IC_{50} value of the recombinant virus was compared to the mean IC_{50} value of the wild-type virus from triplicates, generating the fold change (FC). The mean fold change (mFC) was calculated as the mean FC of independent experiments. SD, standard deviation of the mFC. *P*-value obtained with two-sided student's *t* test. 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; NVP, nevirapine; PFA, foscarnet; TDF, tenofovir.

[b] Slope *k* of regression curve log 10 (fold replication capacity)$=e^{kt}$ and time *t* as intervals of 24 h after 72 h post-inoculation. For parental virus NL4.3 $k=2.15$ and $R^2=0.93$.

human chromosome 17 orf 68, encoding the CTS telomere maintenance complex component 1 (CTC1), as well as with a *pol* insertion observed in a Japanese HIV-1 patient (Sato et al., 1999).

Epidemiological and clinical information did not indicate a direct linkage between the study patient and the Japanese patient. Our patient was an adult Caucasian male who was HIV-1 diagnosed in Belgium in 1993 and was probably heterosexually infected in Sub-Saharan Africa, whereas the Japanese patient was born from a seropositive mother in Japan in 1991 (Sato et al., 1999). The mother was most likely infected by her husband who had a history of sexual contacts with female prostitutes in Thailand in 1989–1990. Furthermore, only the HIV-1 strain from her child developed the insertion after exposure to AZT+ddI, followed by AZT+3TC+NFV/IDV in 1999 (Sato et al., 2001). The phylogenetic analysis confirmed that the two patients could not have infected each other.

Therefore, the detection of an identical human genome sequence from chromosome 17 at an identical location in the HIV-RT of two epidemiological unlinked patients indicates a separate mutation event. It suggests that both sites in the human and viral genome are prone to host sequence transduction and that it provides an evasion mechanism to the virus under therapeutic pressure. The minus-strand exchange model is currently the favored model for retroviral genetic recombination (Onafuwa-Nuga and Telesnitsky, 2009). In this model, RNase H-mediated template degradation exposes the nascent minus strand DNA and enables its subsequent hybridization with complementary regions on the other co-packaged viral RNA (acceptor template). It is suggested that host sequence transduction can occur when the provirus is integrated upstream of the host derived insert-coding region (Onafuwa-Nuga and Telesnitsky, 2009). Read-through of the polyadenylation signal could then lead to the generation of a chimeric retroviral–human RNA that is encapsulated in budding virions. As host sequence transduction mostly reveals the absence of identity between donor and acceptor template behind the junctions, recombination is driven by only short stretches of sequence identity, enabling the transfer of the primer terminus to the acceptor template. Indeed, codon AGC at HIV-1 RT position 68 in our study patient might have facilitated the recombination event as it matches to the NT sequences adjacent to the human genome insertion (Fig. 2). Of interest, AGC is rarely observed in most strains from all subtypes (range from 2.5% to 7.02%), except in

Fig. 3. Phylogenetic analysis of the Belgian and Japanese RT sequences (colored in red and blue, respectively), together with the most similar HIV-1 sequences and with HIV-1 subtype A and CRF01_AE sequences from the local epidemics. Manual phylogenetic analysis was performed using the neighbor joining method (HKY-parameter, bootstrap 1000). The tree was midpoint rooted to allow easier visualization.

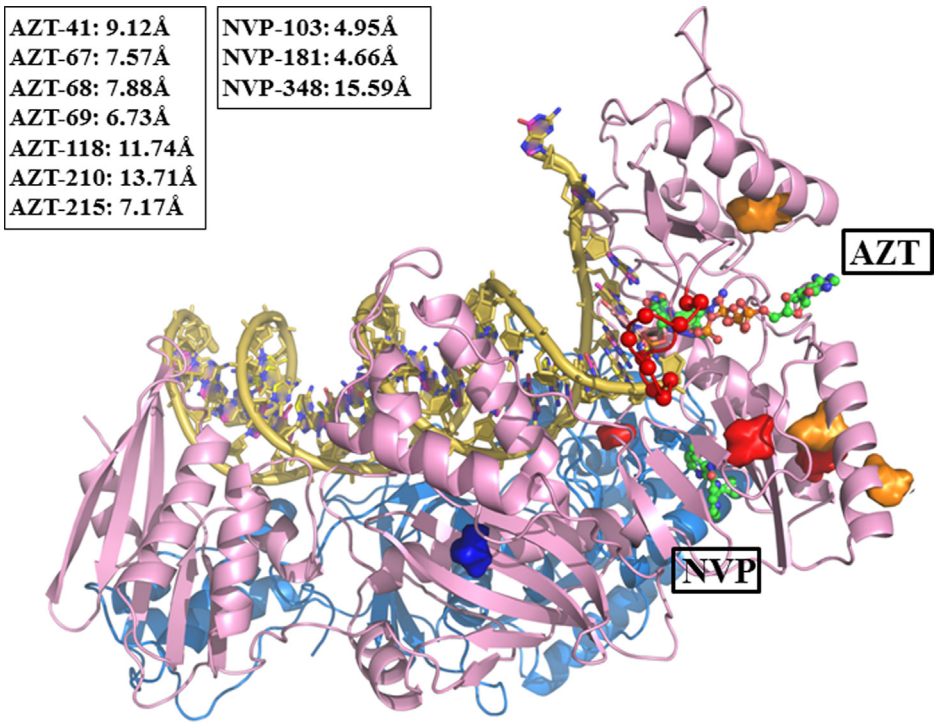


Fig. 4. Mapping of the mutated residues on the HIV-1 RT structure (PDBID: 1RTD) using Yasara (Krieger et al., 2002). The HIV-1 RT p66 unit, p51 unit and the DNA/RNA heteroduplex are colored pink, light blue and yellow, respectively. The structures of AZT and NVP drugs are represented. Mutated residues observed in sample 3, sample 4 or in both samples are colored in blue, red and orange respectively.

subtype A (90.37%), CRF01_AE (79.57%), subtype J (35.14%) and subtype K (25%). As the insertions in both patients differed from the modeled founder sequence at a few nucleotide positions, subsequent rearrangements and point mutations would have contributed to the observed genetic changes.

In conclusion, the *in vivo* retention of the 10-amino acid insertion for more than 6 years suggests that the insertion is well-tolerated. Together with the high-levels of *in vitro* NRTI resistance and replication capacity, the insertion is likely to deliver a selective advantage in the presence of NRTI-containing ART. As the horizontal gene transfer has been observed to occur independently in two unrelated patients carrying different subtypes of HIV-1, it can be considered as a typical example of convergent evolution.

Nucleotide sequence accession numbers

Sequences have been submitted to GenBank under accession numbers KJ438641–KJ438646.

Materials and methods

Patient samples

Plasma and PBMC samples were obtained from a patient attending the AIDS Reference Center at the University of Liège. Plasma viral load was determined using the Amplicor HIV-1 Monitor Test (Roche Diagnostics, Vilvoorde, Belgium).

Cells, compounds and plasmids

Human embryo kidney cells (293T) (ATCC, LGC Standard, Teddington, UK), U87.CD4.CCR5.CXCR4 cells and MT-4 cells were cultivated as previously described (Covens et al., 2009; Miyoshi et al., 1981; Princen et al., 2004).

Zidovudine (AZT), abacavir (ABC), stavudine (d4T), didanosine (ddI), lamivudine (3TC), nevirapine (NVP) and efavirenz (EFV) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Tenofovir (TDF) was kindly provided by Gilead Sciences (Foster City, CA). Foscarnet (PFA) was purchased from Sigma-Aldrich (Diegem, Belgium).

The molecular clone used to generate recombinant viruses was pNL4.3-ΔRT-EGFP (Covens et al., 2009). It displays a deletion from RT codons 1 to 560 and contains the gene encoding an enhanced green fluorescent protein (EGFP) between *env* and *nef* without affecting the expression of any HIV gene.

Extraction, amplification and cloning of viral genome fragments

One milliliter of plasma from clinical samples was ultracentrifuged at 37,100*g* for 1 h and subsequently RNA was extracted using the extraction procedure from the Viroseq HIV-1 Genotyping System (Abbott Molecular, Louvain-La-Neuve, Belgium). DNA was extracted from PBMC samples using the QIAamp DNA minikit (QIAGEN, Venlo, The Netherlands). For recombinant viruses, 20 μl of virus supernatant was lysed with 560 μl AVL buffer (QIAGEN) while incubated for 10 min at room temperature. Subsequently, viral RNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in 50 μl of RNase-free water (Sigma-Aldrich).

Several fragments covering nearly the entire HIV-1 genome were amplified using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Life Technologies, Gent, Belgium) in the outer PCRs and Expand High Fidelity PCR System (Roche Diagnostics) in the inner PCRs (Covens et al., 2009; Snoeck et al., 2005; Van Laethem et al., 2005, 2006, 2008). The RT amplification products were directly inserted into a plasmid using the TOPO XL PCR Cloning Kit (Life Technologies). Single transformed *Escherichia coli* cells were incubated overnight in a shaking incubator at 37 °C using 5 ml LB medium.

Extraction and amplification of human genome fragments

Human genomic DNA was extracted from PBMC samples using the QIAamp DNA Mini Kit (QIAGEN) and a 339-bp fragment was amplified using primers KVL126 (5′-TAG GAA GCG AGG GGG TGA GCA GCG AG-3′) and KVL127 (5′-AGA ACC AGT AAG AGG TAG GAG CGG ACC GAC-3′), Expand High Fidelity PCR System (Roche Diagnostics) and 2 mM $MgCl_2$. The amplification started with an initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 5 min.

Sequencing

PCR products and plasmids were purified with Microspin S-400 (GE Healthcare, Diegem, Belgium) and QIAprep Miniprep Kit (QIAGEN), respectively. Sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Life Technologies) and reactions were run on an ABI3130*xl* Genetic Analyzer (Life Technologies). The sequences were analyzed using Sequence Analysis version 3.7 and SeqScape version 2.0 (Life Technologies) (Covens et al., 2009; Snoeck et al., 2005; Van Laethem et al., 2005, 2006, 2008). Subtype was automatically assigned using Rega Subtyping Tool version 2.0 (de Oliveira et al., 2005).

Phylogenetic and transmission cluster analysis

Highly similar sequences were searched using default settings in nucleotide BLAST (BLASTN) via NCBI (available at http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1997) and the nucleotide collection database (nr/nt, all GenBank+EMBL+DDBJ+PDB sequences). Expect values (*E*) are those assigned by BLASTN using default gap and mismatch penalties. The *E*-value is a parameter that describes the number of hits that can be expected by chance when searching a database of a particular size.

Control PR–RT nucleotide sequences for subtype A and CRF01_AE were downloaded from the Los Alamos HIV Database (http://www.hiv.lanl.gov/), from where one sequence per patient was obtained. Sequences were aligned using ClustalW. Insertion and positions associated with drug resistance were removed from the alignment (Bennett et al., 2009). Phylogenetic analysis was performed using the neighbor joining method in SeaView version 4 (HKY-parameter, bootstrap 1000) (Gouy et al., 2010).

Drug susceptibility and replication capacity

RT-recombinant viruses containing the EGPF gene were generated as previously described (Covens et al., 2009). In brief, 2 μg of purified RT-PCR product (QIAquick PCR Purification Kit, QIAGEN) and 10 μg of purified XbaI-digested pNL4.3-ΔRT-EGFP (PuriLink HiPure Plasmid Filter Purification Kit, Life Technologies) were co-transfected in 293T cells using the standard calcium-phosphate method. Subsequently, the supernatant was transferred to freshly plated U87.CD4.CCR5.CXCR4 cells and cell cultures were monitored for EGFP-expression using fluorescence microscopy. Virus supernatant was harvested using low speed centrifugation and stored at −80 °C for later use. The RT sequence of the recombinant viruses was confirmed as previously described (Covens et al., 2009). In 3 triplicate experiments, drug susceptibilities towards AZT, ABC, d4T, TDF, ddI, 3TC, NVP, EFV and PFA were determined in U87.CD4.CCR5.CXCR4 cells 44 h after infection by measuring EGFP-expression by flow cytometry. Two-sided one-sample *t*-tests ($P<0.05$) were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California, USA).

For the analysis of replication capacity, one million exponentially growing MT-4 cells were infected with recombinant viruses (pre-diluted to achieve 0.1% of EGFP-positive cells after 72 h) in a total volume of 500 μl. The testing was done three times in triplicate for each virus in 24-well plates. After 4 h, the remaining viruses were washed away with PBS and cells were cultured in 2 ml of RPMI to propagate MT-4 cells at 37 °C. Seventy-two hours after infection, the amount of cells was normalized to 750,000 cells/ml and excess cells were fixed with 2% paraformaldehyde. This procedure was repeated every 24 h for 72 h (i.e. 96, 120 and 144 h after infection). The percentage of EGFP-expressing cells was determined using flow cytometry. The amount of infected cells was normalized to the percentage of infected cells at 72 h (day 0). The curves were fitted exponentially and the resulting *k*-values ($y=e^{kt}$) were used as a measure of replication capacity (Auwerx et al., 2008; Brockman et al., 2006). Two-sided two-sample *t*-tests ($P<0.05$) were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software).

Mapping of the insertion on HIV-1 RT

The graphic representations of the HIV-1 RT structure in complex with AZT and NVP were generated using the Yasara program (version 13.2.21) (Krieger et al., 2002). The HIV-1 RT structure (PDBID: 1RTD) was superposed on the structures containing AZT (PDBID: 3V4I) and NVP (PDBID: 4B3Q). Yasara was used to map mutations and the inserted loop using the BuildLoop command. The OptimizeLoop command was run to improve the conformation of the loop. Pymol v1.5 (http://www.pymol.org/) was used to evaluate the structural contacts between the inhibitors (AZT or NVP) and RT mutations calculating the minimum Euclidean distances between atoms of inhibitors and non-hydrogen atoms of residues (Duarte et al., 2010). Contacts were considered direct when the Euclidean distance between inhibitor and residue was less than 8 Å in the RT structure (Morcos et al., 2011).

Acknowledgments

We would like to thank Jan Balzarini and Fossie Ferreira for their assistance. This work was supported by the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Grants G.0611.09N and G.0692.14N), a grant from the Interuniversity Attraction Poles Programme, Belgian State, Belgian Science Policy (IUAP-VI P6/41), the European Community's Seventh Framework Programme (FP7/2007-2013) under the project 'Collaborative HIV and Anti-HIV Drug Resistance Network' (CHAIN, Grant 223131) and the AIDS Reference Laboratory of Leuven that receives support from the Belgian Ministry of Social Affairs through a fund within the Health Insurance System.

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