

Insertion of two amino acids combined with changes in reverse transcriptase containing tyrosine-215 of HIV-1 resistant to multiple nucleoside analogs

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Objective: To identify genotypic drug resistance patterns of HIV-1 in patients who were extensively pretreated with anti-HIV drugs and not responding to their current antiretroviral combination therapy.

Methods: Drug susceptibility of the viruses was tested by a phenotypic recombinant virus assay. Genotypic analysis of HIV resistance was performed by sequencing of the amino-terminal part of the corresponding reverse transcriptase (RT) gene (amino acids 1–280) for serum-derived and recombinant viruses.

Results: Among viruses from 92 patients studied, three (3%) viruses contained a T₂₁₅Y amino-acid change as well as a previously unseen combination of an amino-acid change at codon 67 (N→E/S) and a two amino-acid insertion between codons 68 and 69 of the RT gene of HIV-1. Phenotypic resistance analysis showed high levels of resistance to zidovudine, lamivudine and stavudine (in all patients) and moderate levels of resistance to didanosine and zalcitabine (in two patients), whereas neither serum-derived nor recombinant viruses contained previously known amino-acid changes conferring resistance to didanosine, zalcitabine, lamivudine and stavudine. However, all recombinant viruses contained an insertion of two amino acids between codons 68 and 69 of RT as well as an amino-acid change at codon 67, as was seen in the serum-derived viruses.

Conclusions: Antiretroviral therapy including zidovudine may yield replicating viruses with a two amino-acid insertion in RT in combination with amino-acid changes at codons 67 and 215, which are highly resistant to lamivudine and stavudine on top of zidovudine and have unpredictable susceptibility to didanosine and zalcitabine despite lack of previously reported corresponding resistance-associated amino-acid changes. It is currently unknown what regimens can induce the emergence of this type of multidrug-resistant viruses. This will only be elucidated when resistance assays are capable of detecting these mutants.

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Introduction

Development of drug-resistant mutants is a general problem in antiretroviral treatment of HIV-1-infected individuals [1-3]. The rate of appearance and susceptibility to particular drugs of drug-resistant mutants in individual patients is highly variable and depends on the type and effectiveness of treatment regimens. Assessment of anti-HIV-1 drug resistance before the start and during treatment is therefore advised to assure long-term efficacy of therapy or to change to appropriate alternative regimens, if necessary [1,4]. For the assessment of anti-HIV-1 drug resistance, various phenotypic [5,6] and genotypic [7-9] assays have been developed. Genotypic assays are based on detection of mutations within the *pol* gene of HIV-1 that have been associated with drug resistance. For first-line assessment, however, the phenotypic characterization of susceptibility of HIV-1 to antiretroviral drugs is the method of choice for the determination of resistance-associated mutations in the *pol* gene, because our knowledge of the relationship between virus drug susceptibility and complex genotypic mutational patterns that are often observed in patients under combination therapy is still insufficient.

Introduction of new antiretroviral drugs and drug combinations extends the spectrum of mutations in the HIV-1 *pol* gene that are associated with drug resistance. This requires genotypic assays to be regularly updated. So far, all commercially available genotypic assays for HIV-1 drug resistance are based on detection of the nucleotide substitutions (amino-acid changes) shown to be related to a particular drug-resistant phenotype. In the present study, we identified three patients failing long-term antiretroviral therapy whose viruses acquired an unusual combination of amino-acid changes and an insertion of two amino acids between codons 68 and 69 and were resistant to lamivudine (3TC), stavudine (D4T), didanosine (ddI), and zalcitabine (ddC) despite lack of previously reported corresponding resistance-associated amino-acid changes. We report the genotypic and phenotypic characterization of these viruses.

Materials and methods

Patients

By 1 January 1998, genotypic analysis of HIV resistance by sequencing the amino-terminal part of the RT gene (amino acids 1-280) had been performed for viruses from 228 patients, most of whom had failed antiretroviral therapy. In 144 of the patients, genotypic changes associated with drug resistance against RT inhibitors were found; in 92 (64%) patients, the T₂₁₅Y amino-acid change was found, which was combined with the L₂₁₀W amino-acid change in 60 (42%) patients.

The three patients presented in this study were visiting the Onze Lieve Vrouwe Gasthuis, Location Prinsengracht (patients 1 and 3) or the Academic Medical Centre, University of Amsterdam (patient 2) for clinical and virological follow-up. Patient 1 was a 28-year-old homosexual man known to be HIV-1-positive since June 1995, patient 2 was a 26-year-old homosexual man known to be HIV-1-positive since April 1991, and patient 3 was a 48-year-old homosexual man known to be HIV-1-positive since October 1985. The history of the treatment regimens for the three patients is shown in Fig. 1, and their Centers for Disease Control and Prevention classes and serum HIV-1 RNA levels are shown in Table 1. None of the three patients HIV-1 RNA levels had decreased during the most recent therapy regimens (Table 1) and therefore their viruses were tested for drug-resistant amino-acid changes.

Quantification of HIV-1 RNA

HIV-1 viral RNA levels were determined in EDTA-treated plasma using the NucliSens assay (Organon Teknika, Boxtel, The Netherlands), which has a detection limit of 400 copies/ml with an input of 200 µl plasma.

Phenotypic recombinant virus assay

Drug susceptibility of the viruses infecting the patients was tested using a phenotypic recombinant virus assay (VIRCO NV, Mechelen, Belgium) [5]. Briefly, HIV-1 RNA was extracted from serum, and a 2.2 kb fragment containing the entire HIV-1 protease and RT-coding sequence was amplified by nested reverse transcription PCR (RT-PCR). The pool of protease-RT-coding sequences was then cotransfected into CD4⁺ T lymphocytes (MT4) with the pGEMT3ΔPRT plasmid from which most of the protease (codons 10-99) and RT (codons 1-482) sequences had been deleted. Due to homologous recombination, chimeric viruses containing protease and RT-coding sequences derived from HIV-1 RNA in serum were generated. Using an MT4 cell-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based cell viability assay, the susceptibility [50% effective concentration (EC₅₀)] of the chimeric viruses to the protease inhibitors indinavir, nelfinavir, ritonavir and saquinavir, and the RT inhibitors zidovudine (ZDV), 3TC, ddI, ddC, D4T, and nevirapine (NVP) were determined. According to the manual, a ratio (EC₅₀ recombinant patient virus/EC₅₀ reference IIB virus) above 4 for a certain drug was the criteria of resistance to that drug.

Sequencing serum-derived virus

Viral RNA was isolated from 200 µl serum using guanidine thiocyanate method, as described elsewhere [10,11]. cDNA was obtained after an RT reaction at 42°C for 50 min by using the antisense primer 3'-half RT (5'-TATTTCTGCTATTAAGTCTTTTGATGGGTCA, HxB2 positions 3535-3505). RT reactions

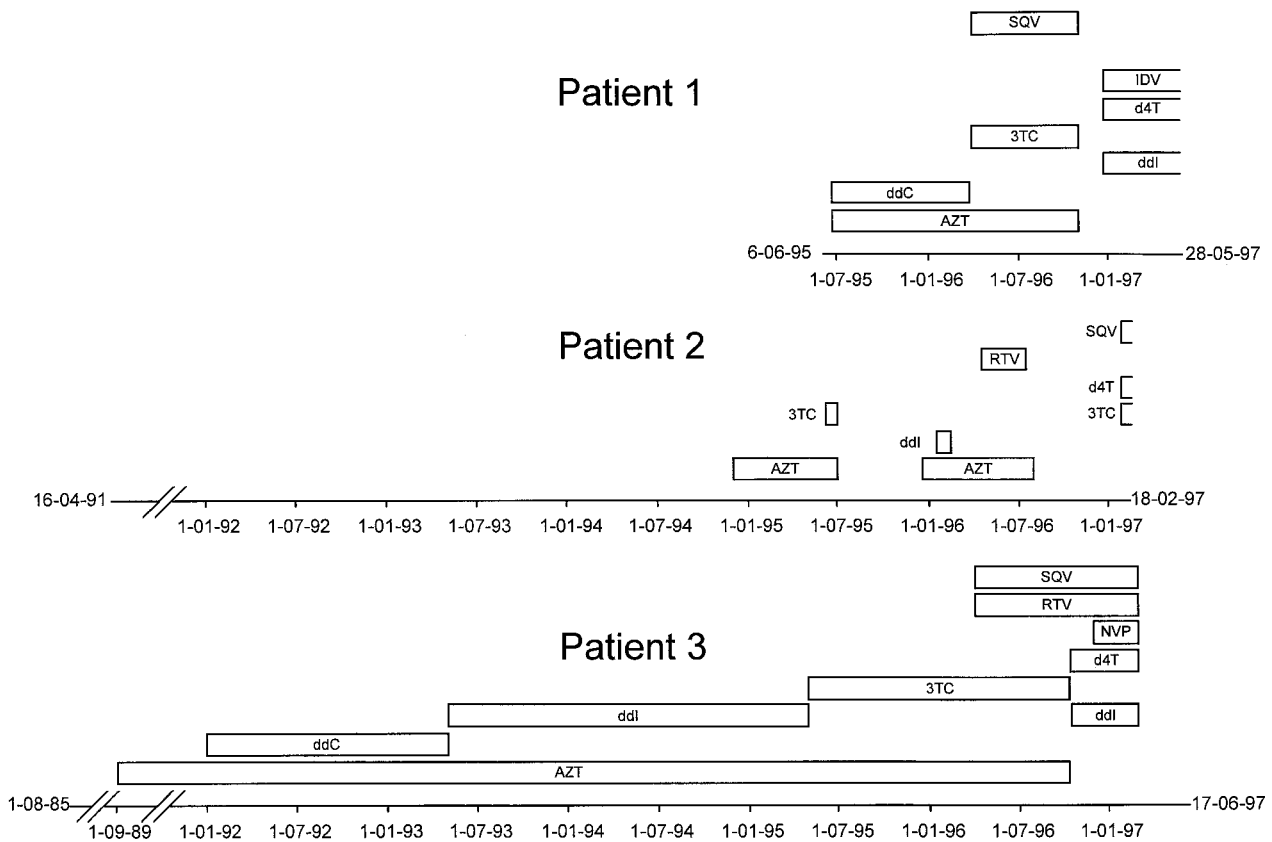


Fig. 1. Anti-HIV-1 drug history of the patients studied. The dates when the viruses were recovered from the patients are shown as the last dates on the time axes.

consisted of 100 ng primer, 250 $\mu\text{mol/l}$ of each dNTP, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.1% Triton, 2.4 mmol/l MgCl_2 , 100 U Superscript II RT (Life Technologies), and 20 U RNAsin in a final volume of 25 μl . Subsequently, a fragment of 1460 base pairs encompassing the protease gene and codons 1–318 of RT was amplified by using the sense primer 5'-PROT-FM (5'-CAAGGGAAGG CCAGGGAA TTT, HxB2 positions 2111–2130) and the antisense primer 3'-half RT. The PCR reaction mixture (final volume 75 μl) was added to the cDNA reaction mixture containing 100 ng of the sense primer 5'-PROT-FM, 183 $\mu\text{mol/l}$ of each dNTP, 20 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.1 mg/ml bovine serum albumin, 2.4 mmol/l MgCl_2 , 2 U *Taq* polymerase (Perkin-Elmer Cetus). The cycle conditions were 95°C

for 5 min, 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and then 72°C for 10 min.

To obtain enough material for sequencing, we subsequently performed two different nested PCR, resulting in fragments designated fragment B or C. For both nested PCR, 5 μl of the first PCR was amplified for 25 cycles in a total reaction volume of 50 μl . The following primers were used for the generation of the two fragments: SP6-5'-P66-OUT (5'-GATTTAGG TGACACTATAGGACCTACACCTGTCAACAT AAT, HxB2 positions 2521–2541) and 3'-END PROT-T7 (5'-TAATACGACTCACTATAGGG AATATTGCTGGTGATCCTTTCCA, HxB2 positions 3065–3041) for fragment B, and 5'-SP6-C-SEQ (5'-GATTTAGGTGACACTATAGGTATACTG

Table 1. Patient characteristics.

	Gender	Age (years)	CDC class	Months on HIV-1 treatment		HIV RNA (copies/ml)	
				Total	Latest	At start of latest treatment	Analysed sample
Patient 1	Male	28	C	23	5	240000	320000
Patient 2	Male	26	A	26	1	160000	410000
Patient 3	Male	48	C	93	2	280000	87000

CDC, Centers for Disease Control and Prevention.

CATTTACCATACC, HxB2 positions 2962–2982) and 3'-half POL-T7 (5' TAATACGACTCACTA TAGGGT CTGCCAGTTCTAGCTCTGCTT, HxB2 positions 3498–3477) for fragment C. The nested PCR reaction mixture contained 50 ng of the primers described above, 200 μ mol/l of each dNTP, 5 μ l of 10 \times Gold PCR buffer II, 1.6 mmol/l MgCl₂, 1.25 U AmpliTaq Gold (Perkin-Elmer Cetus). The cycle conditions were 95°C for 10 min, 25 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and then 72°C for 10 min. The PCR fragments were directly sequenced from the non-coding strand. The nested primers were extended with an SP6 primer sequence (5'-GATTTAGGTGACACTATAGG for the sense primer and with a T7 sequence (5'-TAAT ACGACTCACTATAGGG) for the antisense primers to enable population sequencing with fluorescence-labelled SP6 and T7 primers (*Taq* dye primers, Applied Biosystems, Foster City, California, USA; Thermo Sequenase fluorescence-labelled primer cycle-sequencing kit, Amersham, Little Chalfont, Buckinghamshire, UK). The sequence products were analysed on an automatic sequencer (Applied Biosystems DNA sequencer model 370A and 373A stretch, Foster City, California, USA).

Results

Genotypic resistance analysis

A combination of the ₆₈SS/₆₉V insertion with the ₆₇E/S amino-acid change was found in three (3%) out of 92 patients with the _{T215}Y amino-acid change, or in three (2%) out of 144 patients who had any amino-acid change associated with drug resistance against RT inhibitors. The serum-derived virus of patient 1 showed the ZDV resistance-associated amino-acid changes _{M41}L, _{L210}W and _{T215}Y (Table 2). For patient 2, the virus had the ZDV resistance-associated amino-acid change _{T215}Y. Sequencing the virus obtained from patient 3 revealed the NVP resistance-associated amino-acid changes _{A98}G and _{Y181}C, the ZDV resistance-associated changes _{L210}W, _{T215}Y, as well as the _{A62}V change associated with resistance to multiple nucleoside RT inhibitors [12,13].

In addition to the amino-acid changes described above, serum-derived viruses from all three patients showed the insertion of two amino acids (SS in two patients, SV in one patient) between amino-acid positions 68 and 69 in RT (Table 3). All three of the observed two-amino-acid insertions differed from each other on the nucleotide level (Table 3). In all patients, not previously described amino-acid changes at codon 67 (_{D67}E in two patients, _{D67}S in one patient) were observed. Sequence patterns that were common for the three patients are shown in Table 3.

Phenotypic resistance analysis

Patient 1 had a serum HIV-1 RNA load of 320 000 copies/ml after receiving ddI, D4T and indinavir for 5 months (Fig. 1, Table 1). Phenotypic resistance analysis revealed high (> 10-fold) resistance (EC_{50} recombinant patient virus/ EC_{50} reference IIIB virus) to ZDV, 3TC, ddC and D4T, intermediate (4–10-fold) resistance to ddI, and no resistance to NVP (Table 2). For patient 2, treatment with 3TC, D4T and SQV lasted 1 month, at which point the serum HIV-1 RNA level was 410 000 copies/ml (Fig. 1, Table 1). Phenotypic resistance analysis showed high resistance to ZDV, 3TC, ddI, D4T, intermediate resistance to ddC, and no resistance to NVP (Table 2). For patient 3, resistance analysis was performed 4 months after he had stopped his last regimen (consisting of ddI, D4T, NVP, RTV and SQV) while having a viral load of 87 000 copies/ml (Fig. 1, Table 1). His recombinant viruses showed high fold-resistance to ZDV, 3TC, D4T and NVP, and no resistance to ddC and ddI (Table 2). To confirm whether genotypes of recombinant viruses were identical to serum-derived viruses, we sequenced a fragment of the RT gene (amino acids 1–280) of the recombinant viruses. For all patients, identical amino-acid sequences of their serum-derived and recombinant viruses were observed.

Discussion

In this study, we found viruses with the insertion of two amino acids between positions 68 and 69 (SS or SV, ₆₈SS/₆₉V) and the amino-acid change at codon 67

Table 2. Susceptibility to anti-HIV reverse transcriptase drugs and resistance-associated mutations.

	Zidovudine		Lamivudine		Didanosine		Zalcitabine		Stavudine		Nevirapine	
	EC_{50}	Mutations	EC_{50}	Mutations	EC_{50}	Mutations	EC_{50}	Mutations	EC_{50}	Mutations	EC_{50}	Mutations
Patient 1	> 1493	_{M41} L _{L210} W _{T215} Y	> 13	None	6	None	17	None	> 32	None	3	None
Patient 2	1346	_{T215} Y	36	None	16	None	6	None	33	None	≤ 1	None
Patient 3	1880	_{L210} W _{T215} Y	13	None	4	None	3	None	15	None	1046	_{A98} G _{Y181} C

EC_{50} , 50% effective concentration ratio.

Table 3. Sequence of HIV-1 reverse transcriptase between residues 65 and 70 in combination with resistance-associated mutations.

	41	50	62	65	66	67	68	.	.	69	70	74	75	77	98	100	103	106	108	116	151	181	184	188	190	210	215	219	
Wild-type	M	I	A	K	K	D	S	.	.	T	K	L	V	F	A	L	K	V	V	F	Q	Y	M	Y	G	L	T	K	
ZDV	L	-	-	-	-	N	-	.	.	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y/F	Q/E	
ddl	-	-	-	R	-	-	-	.	.	-	-	V	T	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	
ddC	-	-	-	R	-	-	-	.	.	D	-	V	T	-	-	-	-	-	-	-	-	-	V	-	-	-	C	-	
Stavudine	-	T	-	-	-	-	-	.	.	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Lamivudine	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	V/I	-	-	-	-	-	
Nevirapine	-	-	-	-	-	-	-	.	.	-	-	-	-	-	G	I	N	A	I	-	-	C/I	-	C	A	-	-	-	
ZDV + ddl/ddC	-	-	V	-	-	-	-	.	.	-	-	I	L	-	-	-	-	-	-	Y	M	-	-	-	-	-	-	-	
Patient 1	L	-	-	-	-	E	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W	Y	-	
								AGCGTG																					
Patient 2	-	-	-	-	-	S	-	S	S	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	
								AGCAGT																					
Patient 3	-	-	V	-	-	E	-	S	S	G	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	-	W	Y	-
								TCTAGT																					

Sequence patterns that are common between the three patients are shown in bold type. ZDV, Zidovudine; ddl, didanosine; ddC, zalcitabine.

(D₆₇E or D₆₇S) in three patients who were extensively pretreated with anti-HIV drugs and were not responding to their current antiretroviral combination therapy. Earlier, a virus with a three amino-acid insertion at the same position had been recovered from a single patient [14]. In another study [15], a ZDV-pretreated patient receiving ddI and hydroxyurea had a similar insertion in RT, which was located between amino acids 69 and 70 instead of between amino acids 68 and 69. This difference could be due to peculiarities of sequence alignments or could indicate that there is some flexibility in the location of the insertion in RT.

Phenotypic resistance measurements revealed a high level of resistance [16–18] to 3TC and D4T in the three patients. However, sequence analysis of the serum-derived viruses and recombinant viruses of these patients did not show the 3TC resistance-associated M₁₈₄V and D4T resistance-associated I₅₀T and V₇₅T amino-acid changes. Moreover, in patients 1 and 2, reduced susceptibility for ddI (6- and 16-fold, respectively) and ddC (17- and 6-fold, respectively) was detected by the phenotypic assay without detection of the K₆₅R, T₆₉D, or L₇₄V amino-acid changes.

Recently, it has been demonstrated that in the absence of other drug-resistant amino-acid changes, the T₆₉SSA insertion alone reduces virus susceptibility to ZDV and D4T approximately twofold [19]. The A₆₂V change (found in patient 3) has been shown to reduce ZDV susceptibility by sixfold in combination with T₆₉SSA, without additional impact on susceptibility to D4T [19]. Besides the insertion, the RT genes from the three patients had a D₆₇E or D₆₇S amino-acid change. The D₆₇N change is associated with ZDV resistance, but according to our sequence database and that of Los Alamos [20], Glu or Ser residues at this position have not been previously described. The D₆₇E or D₆₇S changes and perhaps other amino-acid changes observed in the three patients may thus play a role in the insertion of two amino acids in RT and contribute

to the resistance profile observed. Due to the complexity and individual difference in genotypic changes in the three patients, a complete analysis of cooperation between the insertion and particular amino-acid changes at other position requires much more time. At present, it is not possible to assess the precise rate of appearance of the insertion mutants in patients; apparently, this is highly variable for different groups of patients and depends on type and effectiveness of treatment. We found a combination of the ₆₈SS/V₆₉ insertion with the D₆₇E/S amino-acid change in three (3%) out of 92 patients with the T₂₁₅Y amino-acid change, or in three (2%) out of 144 patients with any amino-acid change associated with drug resistance against RT inhibitors. Our study, together with previous reports, indicates that the ₆₈SS/V₆₉ insertion is not an unusual phenomenon during antiretroviral therapy and should therefore be considered in genotypic analysis of antiretroviral resistance in patients.

In summary, our study demonstrates the emergence of HIV-1 strains with an insertion of two amino acids between positions 68 and 69 and a D₆₇E/S amino-acid change. These viruses are resistant to ZDV, 3TC and D4T (and, in two patients, to ddI and ddC) in the absence of previously known amino-acid changes associated with resistance to ddI, ddC, 3TC and D4T. The significance of this finding can only be assessed when drug-resistance assays are able to detect these genotypic changes.

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