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Emergence of human immunodeficiency virus type 1 variants containing the Q151M complex in children receiving long-term antiretroviral chemotherapy

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Abstract

We examined 28 children with HIV-1 infection who were not responding to existing antiviral regimens and were enrolled into clinical trials conducted at the National Cancer Institute to receive salvage therapy. In 3 of the 28 patients (10.7%), the Q151M complex amino acid substitutions were identified. The three patients had received nucleoside reverse transcriptase inhibitor (NRTI) monotherapy and/or combination regimens with multiple NRTIs for 4.3–8.6 years prior to the study. Recombinant infectious clones generated by incorporating the RT-encoding region of HIV-1 isolated from patients' plasma samples were highly resistant to zidovudine, didanosine and stavudine, while they were moderately resistant to lamivudine and tenofovir disoproxil fumarate (TDF). TDF-containing regimens reduced HIV-1 viremia in two of the three children carrying the Q151M complex. These data suggest that the Q151M could be prevalent in pediatric patients with long-term NRTI monotherapy and/or dual NRTI regimens and that HAART regimens containing TDF may be meritorious in such patients.
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1. Introduction

Highly active antiretroviral therapy (HAART) has dramatically improved the quality of life and survival of individuals infected with human immunodeficiency virus type 1 (HIV-1) (Pillay et al., 2000; Lee and Henderson, 2001; Yeni et al., 2002). However, drug-resistant HIV-1 variants often emerge, resulting in treatment failure, which has been a major obstacle in achieving optimal therapeutic efficacy of HAART (Larder and Kemp, 1989; Shirasaka et al., 1993; Shafer et al., 1999; Grabar et al., 2000; Wainberg and Turner, 2004). A set or subset of five substitutions in the viral reverse transcriptase (RT), including Ala62 → Val (A62V), V75I, F77L, F116Y, and Q151M, confers multi-dideoxynucleoside resistance (MDR) on HIV-1 and has been referred to as the 'Q151 complex' (Shirasaka et al., 1993, 1995; Shafer et al., 1994; Schmit et al., 1996; Kavlick et al., 1998; Shulman and Winters, 2003; Zaccarelli et al., 2004). The frequency of the emergence of the Q151M complex-associated amino acid substitutions appears to be relatively low, ranging from 1.9% to 6.25% depending on the cohorts examined (Lawrence et al., 1999; Rousseau et al., 2001; Englund et al., 2004; Zaccarelli et al., 2004), although a few studies have shown a relatively higher frequency ranging from 8.8% to 17% (Kavlick et al., 1998; Re et al., 2003). The reason for the high frequency observed in such cohorts appears to be that those individuals received nucleoside reverse transcriptase inhibitor (NRTI) monotherapy and/or dual NRTI combination therapy for substantially longer periods of time in

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the pre-HAART era (Kavlick and Mitsuya, 2001; Yin et al., 2006).

To our knowledge, there are only two works reporting the emergence of HIV-1 containing the Q151M complex in children with HIV-1 infection (Englund et al., 2004; Hazra et al., 2005), but no detailed study of such Q151M-carrying HIV-1 variants has been conducted. A previous study by Palumbo et al. (2001) has shown no significant association between perinatal transmission and the presence of zidovudine (ZDV) or NRTI resistance-associated mutations. They also showed that the mutation patterns identified in children were not identical to those of their mothers. Thus, it is possible that HIV-1 variants carrying the Q151M complex are not readily transmitted. It is also possible that the number of pediatric cases examined for the presence of drug-resistant HIV-1 variants is too limited and that the occurrence of drug-resistant HIV-1 variants has been overlooked. In the present study, we examined HIV-1 variants isolated from 28 children with HIV-1 infection who were enrolled into the phase I/II study of tenofovir disoproxil fumarate (TDF) (Hazra et al., 2005) and a study of therapeutic drug monitoring and viral resistance testing. The present work represents the first extensive study to characterize the virological profiles of Q151M-carrying HIV-1 variants isolated from children receiving HAART.

2. Materials and methods

2.1. Patients

Twenty-eight children with AIDS, who had been on long term anti-HIV therapy and were not responding to existing regimens of combinations of anti-HIV-1 agents, were enrolled into one of the following two clinical trials to receive salvage therapy: a phase I/II study of TDF (Hazra et al., 2005) and a study of therapeutic drug monitoring and viral resistance testing (R.H., personal communication). Subjects on the former study must have failed at least two prior regimens to be eligible, and subjects on the latter study could be receiving their first HAART regimen on the study, but most had failed several previous regimens. On the phase I/II study of TDF, children received TDF-containing HAART, while on the therapeutic drug monitoring study, they could have received TDF but did not have to. Parents or guardians of the subjects agreed to and signed the informed consent form. The Institutional Review Board of the National Cancer Institute approved the study.

2.2. Cells

MT-2 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT), 50 U of penicillin/ml, and 50 μg of streptomycin/ml. Peripheral blood mononuclear cells (PBMC) obtained from HIV-1-negative healthy donors were stimulated with phytohemagglutinin (PHA) in RPMI 1640-based medium containing recombinant interleukin-2 (5 ng/ml; R&D Systems, Minneapolis, MN) for 2 days (PHA-PBMC) prior to HIV-1 exposure. HIV-1 strains were isolated as previously described (Shirasaka et al., 1993) by culturing PBMC obtained from patients, passaged once or twice in PHA-PBMC, and stored at −80 °C until used.

2.3. Determination of nucleotide sequence of HIV-1

HIV-1 RNA was isolated from plasma samples of the patients as previously described (Tamiya et al., 2004). In brief, viral RNA was extracted from plasma samples by using QIAamp Viral RNA minikit (Qiagen, Valencia, CA) and the RT-encoding region of HIV-1 was amplified using RT-PCR with QIAGEN OneStep RT-PCR kit (Qiagen). Thus obtained PCR products were directly sequenced using Applied Biosystems model 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

2.4. Generation of molecular infectious HIV-1 clones containing RT of clinical HIV-1 isolates

Molecular infectious HIV-1 clones carrying RT of HIV-1 were generated as previously described (Gatanaga et al., 2002; Tamiya et al., 2004). In brief, viral RNA extracted from plasma samples was subjected to RT-PCR using OneStep RT-PCR kit (Qiagen) to amplify the RT-encoding region of HIV-1. Primers used were EagI-1 (5′-CAACTCCCTCTCAGAAGCAGCGGCCGATAGACAAGGAACTG-3′) and EcoRI-R (5′-CAGCAGTTGTTGCAGAATTCT TATTATGGCTTCCAC - 3′). RT-PCR products (1 μl) were used directly in the nested-PCR with XmaI-F (5′-CCAGTAAAATTAAAGCCCGGGATGGATGGCCCAAAAG-3′) and ClaI-R (5′-GTTAAAATCACTAGCCATCGATCTCCAATTACTGTG-3′). XmaI-F primer had an *Xma*I site, whereas ClaI-R primer a *Cla*I site. The PCR products obtained were digested with *Xma*I and *Cla*I, and the largest fragments were introduced into pHIV-1_{NL4-3} (Tamiya et al., 2004). Each recombinant plasmid was transfected into COS-7 cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the infectious virions obtained were harvested 48 h after transfection and stored at −80 °C until use. Thus obtained molecular infectious HIV-1 clones containing the RT region (aa 21–560) of HIV-1 from Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} were designated as rHIV-1_{ERS1}, rHIV-1_{ERS2}, and rHIV-1_{ERS3}, respectively. Titration assays for these molecular infectious clones obtained were titrated as previously described (Shirasaka et al., 1993; Reed and Muench, 1938) in six replicates.

2.5. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to determine the cytopathic effect (CPE) in MT-2 cells exposed to each virus preparation as previously described (Gatanaga et al., 2002; Matsumi et al., 2003). All assays were performed in triplicate.

2.6. Drug sensitivity assays

The sensitivities of the rHIV-1_{NL4-3} and infectious molecular HIV-1 clones to various drugs were determined as previously described with minor modifications (Shirasaka et al., 1995).

Briefly, MT-2 cells (2×10^3) were exposed to 100 $CCID_{50}$ 50% cell culture infectious dose of rHIV-1_{ERS1}, rHIV-1_{ERS2}, or rHIV-1_{ERS3} and were cultured in the presence of various concentrations of each reverse transcriptase inhibitor (RTI) in 96-well microculture plates and incubated at 37 °C for 7 days. The 50% inhibitory concentration (IC_{50}) values were determined using the MTT assay in triplicate. The value in parentheses represents a fold difference of the IC_{50} value of each RTI against each recombinant clone as compared to the IC_{50} value against rHIV-1_{NL4-3}.

The IC_{50} values were determined using the target PHA-PBMC exposed to HIV-1_{LAI} or HIV-1_{ERS2} (50 $CCID_{50}/10^5$ PBMC) in the presence of each RTI in 96-well microtiter culture plates and the inhibition of p24 Gag protein production by the drug as an endpoint, as previously described (Shirasaka et al., 1995). All values were determined in triplicate and those shown are representative of three separate experiments. The numbers in parentheses represent fold differences compared to the IC_{50} against HIV-1_{LAI}.

3. Results

3.1. Amino acid substitutions in the RT-encoding region of the genome of HIV-1 isolated from pediatric patients

Viral RNA samples isolated from patients' plasma samples were subjected to RT-PCR for identifying amino acid substitutions in the RT-encoding region of HIV-1 genome. Approximately 700 bp of the *pol* gene, spanning codons 30–260 of RT, were determined in all HIV-1 samples. Table 1 shows all amino acid substitutions identified, which have been reported to associate with drug resistance as we examined the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu) (Shafer et al., 1999; Rhee et al., 2003). Among them were the M184V substitution in 13 of the 28 HIV-1 isolates (46%), K70R in 8 of 28 (29%), T69D in 7 of 28 (25%), and V118I in 6 of 28 (21%). The 69 insertion complex conferring multidideoxynucleoside resistance was also identified in 2 of 28 (7%). It was noted that of the 28 patients, three patients (Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3}: 10.7%) were found to harbor HIV-1 that contained one or more of the Q151M complex substitutions (Shirasaka et al., 1993, 1995; Shafer et al., 1994) (Table 1). In the viruses isolated from 3 of 28 patients (10.7%), only amino acid substitutions that are not known to confer detectable resistance on HIV-1 were identified.

3.2. Amino acid substitutions identified in the protease-encoding region of HIV-1 genome

All of the 28 HIV-1 isolates were found to contain amino acid substitutions in the protease-encoding region, which have been shown to be associated with viral resistance against protease inhibitors (PIs) (Shafer et al., 1999; Rhee et al., 2003), as illustrated in Table 1. Among them were L90M (Eberle et al., 1995) in 15 of 28 (54%), V82A (Molla et al., 1996) in 14 of 28 (50%), and M46I (Kaplan et al., 1994) in 9 of 28 (32%). Isolates from 3 of 28 patients (10.7%) had no significant amino acid substitutions in the protease-encoding region, although there were substitutions that are not known to confer detectable resistance on HIV-1.

3.3. Clinical profiles of three children harboring HIV-1 that contained one or more of the Q151M complex

Clinical profiles of the three patients who harbored HIV-1 variants containing one or more of the Q151M-associated sub-

Table 1
Amino acid substitutions identified in HIV-1 isolates from 28 pediatric patients

Amino acid substitutions in RT	No. of patients	Amino acid substitutions in protease	No. of patients
Q151M complex	3	D30N	2
E44D, M184V	1	D30N, M46I	1
E44D, T69D, 184V	1	D30N, L90M	1
E44D, T69D, V118I, M184V	1	M46I	1
E44D, T69D, V118I, Y181C	1	M46I, V82A	1
E44D, K70R, M184V, T215Y	1	M46I, V82A, I84V, L90M	1
T69D, K70R	2	M46I, V82A, L90M	2
T69D, K70R, M184V	1	M46I, V82S, L90M	1
T69D, M184V, G190A	1	M46I, V82T, L90M	1
K70R, K103N, V118I, Y181C	1	M46I, I84V, L90M	1
K70R, M184V	1	V82A	3
K70R, M184V, T215Y	1	V82A, I84V, L90M	1
K70R, T215Y	1	V82A, L90M	6
L74V, K103N, V118I, M184V, T215Y	1	I84V	1
L74V, V118I, M184V, G190S	1	L90M	2
L74V, Y181C, Y188L	1	No primary mutations identified	3
K103N	1		
K103N, V108I, M184V, T215Y	1		
V108I, V118I, M184V	1		
M184V	1		
T69S + SG insertion	2		
No primary mutations identified	3		

Table 2
Clinical profiles of Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3}

Patient	Sex	Age	$CD4^+$ (cells/mm^3)	HIV-1 RNA (copies/ml)	Year on antiviral therapy
Pt_{ERS1}	Female	11.3	42	150,000	8.6
Pt_{ERS2}	Male	10.3	2	544,000	7.6
Pt_{ERS3}	Male	10.7	0	364,000	4.3

stitutions, are shown in Table 2. The three subjects (two males and one female) were 10 or 11 years old, were severely immunocompromised, and had very low $CD4^+$ T cell counts. Pt_{ERS2} was first treated with ZDV monotherapy, followed by administration of ZDV + lamivudine (3TC), and later with various PIs and non-nucleoside RT inhibitors (NNRTIs), as shown in Fig. 1. Pt_{ERS1} initially received didanosine (ddI) monotherapy, followed by ddI + stavudine (d4T) combination, and later received various drugs, while Pt_{ERS3} initially received ZDV + 3TC combination and subsequently ddI, d4T, nevirapine (NVP), and ritonavir (RTV). It is of note that all these three patients were initially treated with NRTI monotherapy or NRTI combination therapy and received one or more NRTIs throughout the treatment periods of 4.3–8.6 years together with other classes of antiviral agents.

The earliest available $CD4^+$ T cell counts of Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} were 473, 288, and 62 mm^{-3}, respectively. Although various antiviral regimens had been administered, CD4 counts had continued to decline until their enrolment into the present salvage therapy trial (Hazra et al., 2005). In the present salvage therapy trial, Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} received ZDV/3TC/saquinavir (SQV)/ritonavir-boosted lopinavir (LPVr)/enfuvirtide (ENF)/TDF, ZDV/3TC/d4T/LPVr/TDF, and ZDV/emtricitabine (FTC)/EFV/RTV/SQV/atazanavir (ATV)/TDF, respectively. With the implementation of TDF-containing therapy, in Pt_{ERS1} and Pt_{ERS2}, CD4 cell counts increased up to 576 and 1018 mm^{-3} and stayed at these levels, and HIV-1 RNA copies decreased by 5.7 and 5.5 logs, respectively. In Pt_{ERS3}, there were no changes in CD4 counts or HIV-1 RNA copies, but with ENF administration, a significant decrease in HIV-1 RNA copy number by 3 logs was achieved, although no increase in CD4 counts occurred (Fig. 1). It is of note that there was a rebound seen later in HIV-1 RNA copy numbers in Pt_{ERS1} and Pt_{ERS3}.

3.4. Genotypic analysis of HIV-1 isolates containing the Q151M complex

HIV-1 isolated from Pt_{ERS1} had all five Q151M complex substitutions (A62V, V75I, F77L, F116Y, and Q151M), while Pt_{ERS2} had four amino acid substitutions (A62V, V75I, F116Y, and Q151M), and Pt_{ERS3} had two substitutions (F116Y and Q151M). These HIV-1 isolates also had substitutions associated with resistance to NRTIs and non-nucleoside

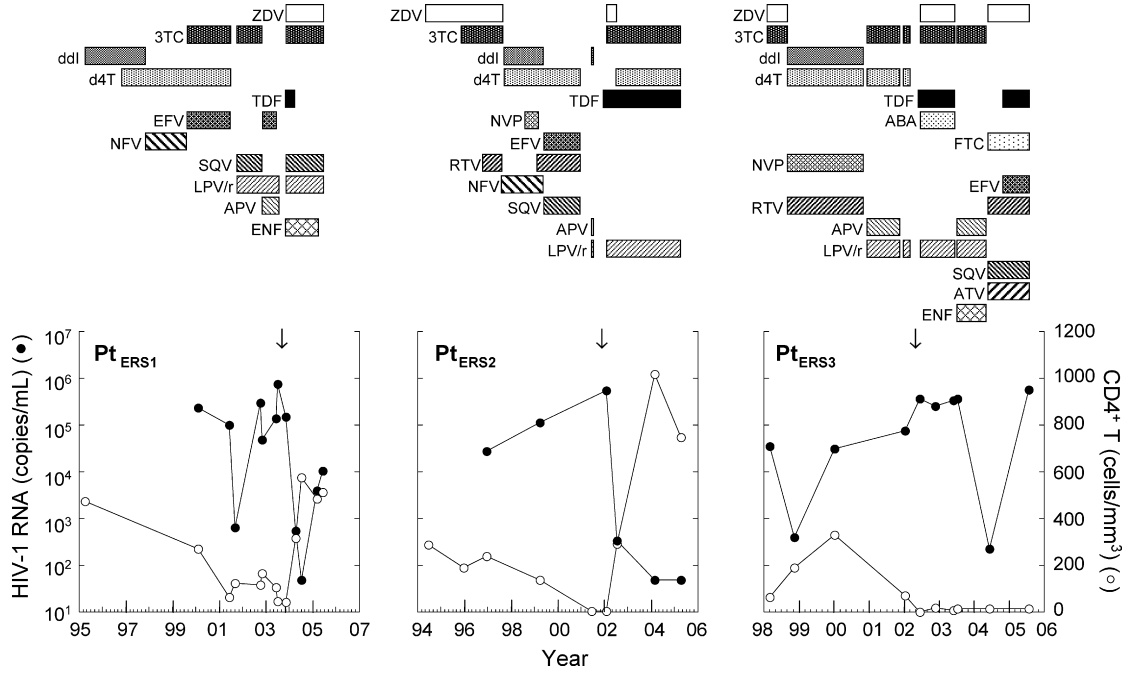

Fig. 1. $CD4^+$ cell counts and HIV-1 RNA copy numbers and the antiviral regimens administered. $CD4^+$ cell counts, HIV-1 RNA copy numbers in plasma, and antiviral regimens the three patients received are illustrated. Each arrow indicates the date of study entry when blood samples were drawn from patients that were subjected to virological analysis reported in this work. It is of note that there is uncertainty in the treatment regimens Pt_{ERS1} received from 1998 to 1999 due to conflicting reports from her clinic record. ZDV, zidovudine; ddI, didanosine; d4T, stavudine; 3TC, lamivudine; RTV, ritonavir; SQV, saquinavir; APV, amprenavir; NFV, nelfinavir; LPV/r, ritonavir-boosted lopinavir; NVP, nevirapine; EFV, efavirenz; TDF, tenofovir disoproxil fumarate; ABA, abacavir; FTC, emtricitabine; ATV, atazanavir; and ENF, enfuvirtide.

Table 3
Amino acid substitutions identified in HIV-1 isolated from the 3 patients and the mother of one of them

Patient	MDR substitutions in RT[a]					Other substitutions in RT[a]											
	A62	V75	F77	F116	Q151	D67	T69	K70	K103	Y181	M184	G190	H208	L210	F214	T215	K219
Pt_{ERS1}	V	I	L	Y	M	–	–	N	–	–	–	A	H/Y[b]	V/F[b]	–	–	–
Pt_{ERS2}	V	I	–	Y	M	N/S[b]	–	–	–	I	–	E	–	–	L	V	Q
Mother of Pt_{ERS2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pt_{ERS3}	–	–	–	Y	M	E/del[b]	G	K/R[b]	N	I	–	–	–	–	L/M[b]	V	E

[a] Amino acids and their positions in HIV-1_{LAI} are shown as a reference at the top; "–" indicates that the amino acid is the same as in the reference sequence.
[b] Amino acids seen mixed. del: deletion.

reverse transcriptase inhibitors: Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} had K70N/G190A/H208Y/L210V(F), D67N(S)/Y181I/G190E/F214L/T215V/K219Q, and D67E(del)/T69G/K70R/K103N/Y181I/F214L(M)/T215V/K219E, respectively. Despite the history of long-term or intermittent 3TC administration in these three patients, no amino acid substitutions were identified at position 184. In this respect, at the time of enrollment and sample collection from Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3}, 3TC administration had been discontinued for 12, 52, and 4 months, respectively (Fig. 1). Thus it is likely that 3TC resistance-associated amino acid substitutions, if they existed, had disappeared from peripheral blood in these patients (Kavlick and Mitsuya, 2001). It is worth noting that two patients had T215V together with the Q151M complex. Although we attempted to determine whether the Q151M complex-containing HIV-1 was transmitted to these children from their mothers, only one plasma sample from mother of Pt_{ERS2} was available. The HIV-1 of the mother of Pt_{ERS2} was isolated from her blood taken in July 2004, when the child was enrolled into the clinical trial. She was found to harbor HIV-1 lacking significant resistance-associated substitutions and the Q151M complex (Table 3).

All three HIV-1 isolates were found to contain primary substitutions associated with PI resistance. These substitutions were thought to be highly related to PIs each patient received during antiviral therapy. Among them were M46I (Kaplan et al., 1994) in HIV-1_{ERS3}, V82A (Molla et al., 1996) in HIV-1_{ERS2} and HIV-1_{ERS3}, and L90M (Eberle et al., 1995) in all three isolates (Table 4).

The Gag-encoding gene contained genetic alterations including a 453-PEPTAP-459 insertion in HIV-1_{ERS2} and HIV-1_{ERS3}, 127-SS-128 insertion plus 472-deletion in HIV-1_{ERS3}.

3.5. Susceptibility of the Q151M-containing HIV-1 variants to RTIs

We propagated a clinical HIV-1 isolate from Pt_{ERS2} by culturing his PBMC with PHA-PBMC from an HIV-1-negative, healthy donor. However, we failed to propagate HIV-1 using samples from Pt_{ERS1} and Pt_{ERS3}. Thus, to study the replication characteristics of these patients' viruses in the presence and absence of antiviral agents, we generated recombinant infectious clones by incorporating the RT-encoding region and a part of integrase-encoding region (aa 21–560 plus aa 1–110) into pHIV-1_{NL4-3} and examined each infectious clone for its susceptibility against various NRTIs and NVP (Table 5). The recombinant infectious clones were confirmed to have the RT-region containing the Q151 complex mutations as well as other mutations (D67N, K70R, and K219Q/E) shown in Table 3. As expected, all three recombinant infectious clones were substantially resistant to ZDV, ddI, and d4T as previously described for other Q151M-containing infectious clones (Shirasaka et al., 1993, 1995). Each clone was only moderately resistant to 3TC with 5–6 fold increase in IC_{50} values in agreement with previous reports (Shirasaka et al., 1993, 1995; Shafer et al., 1994). All three clones (rHIV-1_{ERS1}, rHIV-1_{ERS2}, and rHIV-1_{ERS3}) were sensitive or only slightly resistant to TDF with 2–4.2-fold increase in IC_{50} values, which probably explain why the regimens containing TDF effectively reduced peripheral HIV RNA levels in Pt_{ERS1} and Pt_{ERS2}. The reason no reduction in HIV-1 RNA copies was seen in Pt_{ERS3} is not clear (Fig. 1). Two clones (rHIV-1_{ERS2} and rHIV-1_{ERS3}) and HIV-1_{ERS2}, all of which contained NVP resistance-associated amino acid substitutions K103N and/or Y181I (Table 3), proved to be highly resistant to NVP, in agreement with previous reports (Richman et al., 1991, 1994).

Table 4
Genetic alterations in protease and Gag of HIV-1 isolated from the 3 patients and the mother of one of them

Patient	Substitutions in protease[a]													Alterations in Gag[a]		
	L10	K20	V32	L33	M36	M46	I54	K55	L63	A71	V77	V82	L90	Q127	P453	P472
Pt_{ERS1}	–	R	I	–	I	–	–	–	P	V	–	L	M	–	–	–
Pt_{ERS2}	–	–	–	–	–	–	–	–	P	V	I	A	M	–	*ins*PEPTAP[b]	–
Mother of Pt_{ERS2}	I	–	–	–	–	I	V	–	P	–	I	–	–	–	–	–
Pt_{ERS3}	F	–	–	F	–	I	V	R	P	V	–	A	M	*ins*SS[b]	*ins*PEPTAP[b]	del

[a] Amino acids and their positions in HIV-1_{LAI} are shown as a reference at the top; "–" indicates that the amino acid is the same as in the reference sequence.
[b] An insertion identified. del: deletion.

Table 5
Susceptibility against RTIs of various infectious clones and HIV-1_{ERS2}

HIV-1	IC_{50} (μM)					
	ZDV	ddI	d4T	3TC	TDF	NVP
rHIV-1_{NL4-3}[a]	0.011 (1)	1.6 (1)	0.36 (1)	1.0 (1)	0.88 (1)	0.091 (1)
rHIV-1_{ERS1}[a]	0.44 (40)	52 (33)	7.2 (20)	6.1 (6)	1.8(2)	0.2 (2)
rHIV-1_{ERS2}[a]	0.15 (14)	62 (39)	5.6 (15)	5.5 (5.5)	3 (3.4)	>10 (>110)
rHIV-1_{ERS3}[a]	0.14 (13)	64 (40)	4.3 (12)	5.2 (5.2)	3.7 (4.2)	>10 (>110)
HIV-1_{LAI}[b]	0.004 (1)	0.69 (1)	0.09 (1)	0.3 (1)	0.06 (1)	0.017 (1)
HIV-1_{ERS2}[b]	0.046 (12)	14 (20)	1.1 (12)	0.9 (3)	0.2 (3.3)	>10 (>588)

[a] MT-2 cells were exposed to rHIV-1_{ERS1}, rHIV-1_{ERS2}, or rHIV-1_{ERS3} and IC_{50} values were determined using the MTT assay. The value in parentheses represents a fold difference of the IC_{50} value of each RTI as compared to that against rHIV-1_{NL4-3}.

[b] The IC_{50} values were determined using PHA-PBMC exposed to HIV-1_{LAI} or HIV-1_{ERS2} in the presence of each RTI and the inhibition of $p24^{Gag}$ protein production by the drug as an endpoint. The numbers in parentheses represent fold differences compared to the IC_{50} against HIV-1_{LAI}.

4. Discussion

In the present study, we identified a set or subset of the Q151 complex (A62V, V75I, F77L, F116Y, and Q151M), which confers MDR on HIV-1 in 3 of 28 (10.7%) children enrolled into a phase I/II study of TDF (Hazra et al., 2005) and a study of therapeutic drug monitoring and viral resistance testing. This rate of the emergence of the Q151M complex is greater than the rates reported by others. For example, in a study conducted between 1991 and 1995 by Englund et al., of 52 children with AIDS who were drug-naïve or had received antiviral therapy for less than 6 weeks and subsequently received ZDV, ddI or both drugs combined for 8.6 months (range, 2.6–28.9 months), only one child developed Q151M (1.9%) (Englund et al., 2004). Larder et al. reported that of 180 patients who had <300 $CD4^+$ cells per mm^3 and had received <4 weeks (range, 0–0.93 months) of ZDV therapy and subsequently ZDV monotherapy or combination of ZDV plus ddI or ZDV plus zalcitabine for 48 weeks, none developed the Q151 complex (Larder et al., 1996). The duration of treatment with NRTI regimens varied from 2.6 to 28.9 months in these studies, but was shorter than the duration of NRTI-containing therapy administered to the 28 pediatric patients of the present study. The duration of therapy for the pediatric patients ranged from ∼4.8 to ∼13.5 years (mean, 9.7 years), so that these patients had received antiviral therapy with multiple NRTIs for significantly longer periods of time, which may relate to the high rate of the emergence of the Q151M complex seen in the present study. It is of note that the duration of treatment with NRTI-monotherapy or dual combination regimens in patients who developed the Q151M complex in our previous study which enrolled those who had received >36 months of ZDV plus ddI (Kavlick et al., 1998) was >52 to >93 months (mean: 68.3 months or longer), and of 36 such patients, 6 patients (17%) were found to harbor HIV-1 variants containing the Q151M complex. Zaccarelli et al., between June 1999 and December 2002, examined as many as 470 patients who had treatment failure and found the Q151 complex in 17 patients, a relatively low ratio of 3.6%. However, these authors found a high association of the emergence of the Q151M complex with younger age, lower CD4 counts, greater HIV-1 RNA copy numbers, and treatment with >2 years prior antiviral therapy (Zaccarelli et al., 2004). Considering that all three patients in the present study are at younger ages with lower CD4 counts and substantially high HIV-1 RNA copy numbers, and >4.3 years of therapy, these factors may possibly explain why the high ratio of the Q151M emergence was seen in our cohort. It is apparent that long-term NRTI monotherapy and/or dual NRTI combination therapy, without other classes of antiviral agents, might be the reason for the high incidence of the Q151M emergence. Another possible reason is that HIV-1-infected children, especially young children, have higher viral loads than adults do. Hence, it is harder to drive the viral loads to undetectable levels with HAART, so that some residual viral replication will continue, even during HAART, rendering it more likely that resistance will develop (Shearer et al., 1997), although further studies are needed to definitively determine the factor(s) responsible for the emergence of the Q151M complex.

In the RT sequence from Pt_{ERS1}, all five substitutions (A62V, V75I, F77L, F116Y, and Q151M) were identified, while Pt_{ERS2} had A62V, V75I, F116Y, and Q151M, and Pt_{ERS3} had F116Y and Q151M (Table 3). However, these two latter HIV-1 populations (Pt_{ERS2} and Pt_{ERS3}) had ZDV resistance-associated substitutions (T215V plus K219Q or K219E) (Table 3). While several previously reported cases of HIV-1 having the Q151M substitution carried both the Q151M complex and a substitution at position 215, such dual mutations are relatively rarely found (Shirasaka et al., 1993, 1995; Kavlick et al., 1998). Indeed, in the Stanford HIV Drug Resistance Database (Shafer et al., 1999; Rhee et al., 2003) compiled to date, of 2642 cases in which a substitution(s) at position 215 was identified, only 33 cases (1.25%) have been found to carry both 215 substitution(s) and the Q151M complex mutation(s). The T215V substitution has been reported in an HIV-1-infected child who developed ZDV resistance with D67N/K70R/T215V/K219Q, although its virologic significance is not completely understood (Orlandi et al., 1998). The examination of the Stanford University HIV Drug Resistance Database revealed that of 2642 persons having substitutions at position 215, only 57 persons had T215V (2.2%), suggesting that although T215V substitution could be related to ZDV resistance, the level of its contribution to ZDV resistance and/or viral replication appears to be low in comparison with that of T215Y (∼73.8% seen in the Database) and T215F (∼20% in the Database). It is not clear at this time as to why Pt_{ERS2} and Pt_{ERS3} had these dual amino acid substitutions.

We generated recombinant infectious HIV-1 clones from all three patients by incorporating into HIV-1_{NL4-3} the RT-encoding region of HIV-1 using viral RNA from their plasma, and examined each clone for its susceptibility to various NRTIs and NVP. In this study, however, we chose not to incorporate amino acid substitutions in protease, since the introduction of amino acid substitutions in protease without amino acid substitutions seen in Gag proteins often lead to greatly compromised replicative ability of the resultant recombinant infectious clones (Gatanaga et al., 2002). We found that all three infectious clones (rHIV-1_{ERS1}, rHIV-1_{ERS2}, and rHIV-1_{ERS3}) were substantially resistant to ZDV, ddI, and d4T, and only moderately resistant to 3TC in agreement with previous reports (Shirasaka et al., 1993, 1995; Shafer et al., 1994). These three clones were sensitive or only slightly resistant to TDF, which probably explains why the TDF-containing regimens reduced the numbers of HIV RNA copy numbers in Pt_{ERS1} and Pt_{ERS2}. However, no reduction occurred in Pt_{ERS3} with the implementation of TDF-containing regimen (Fig. 1). In this regard, Pt_{ERS3} had previously been reported to have the K65R substitution that has been shown to be associated with viral resistance to TDF (Wainberg and Turner, 2004), although K65R was not detected at baseline in the present study. Thus, re-emergence of K65R-containing variants may explain his lack of response. It is also of note that Pt_{ERS3} was intermittently non-adherent to the salvage regimen. Such non-adherence might have been the major reason for his treatment failure.

HIV-1 with mutations conferring drug resistance are likely less fit than the wild-type virus and are transmitted relatively inefficiently (Leigh Brown et al., 2003), although there is a growing body of literature indicating increasing cases of transmission of drug-resistant HIV-1 variants (Salomon et al., 2000; Masquelier et al., 2005), posing a formidable threat to those at risk. Nevertheless, there has been no report to date that HIV-1 carrying the Q151M complex was transmitted in the form of either sexual or vertical transmission. Thus, we attempted to ask whether the Q151M complex was transmitted from their mothers to the children. However, only a blood sample from Pt_{ERS2}'s mother was available and this mother's HIV-1 had no significant amino acid substitutions in RT or protease (Tables 3 and 4).

In conclusion, of 28 pediatric patients examined in the present study, the Q151M complex was identified in three patients (10.7%), all of who had received ZDV monotherapy and/or combination regimens with multiple NRTI over 4.3–8.6 years. This work represents the first extensive study to characterize the virological profiles of Q151M-carrying HIV-1 variants isolated from children receiving HAART and suggests that the Q151M emergence rate could be high in pediatric patients who are treated with long-term NRTI monotherapy and/or dual NRTI regimens. The present data also suggest that TDF- or ENF-containing regimens may be meritorious in such patients with the Q151M complex.

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