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Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients

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ABSTRACT

Some mutations in the connection subdomain of the polymerase domain and in the RNase H domain of HIV-1 reverse transcriptase (RT) have been shown to contribute to resistance to RT inhibitors. However, the clinical relevance of such mutations is not well understood. To address this point we determined the prevalence of such mutations in a cohort of antiretroviral treatment-naïve patients ($n = 123$) and assessed whether these substitutions are associated with drug resistance *in vitro* and *in vivo*. We report here significant differences in the prevalence of substitutions among subtype B, and non-subtype B HIV isolates. Specifically, the E312Q, G333E, G335D, V365I, A371V and A376S substitutions were present in 2–6% of subtype B, whereas the G335D and A371V substitutions were commonly observed in 69% and 75% of non-B HIV-1 isolates. We observed a significant decline in the viral loads of patients that were infected with HIV-1 carrying these substitutions and were subsequently treated with triple drug regimens, even in the case where zidovudine (AZT) was included in such regimens. We show here that, generally, such single substitutions at the connection subdomain or RNase H domain have no influence on drug susceptibility *in vitro* by themselves. Instead, they generally enhance AZT resistance in the presence of excision-enhancing mutations (EEMs, also known as thymidine analogue-associated mutations, TAMs). However, N348I, A376S and Q509L did confer varying amounts of nevirapine resistance by themselves, even in the absence of EEMs. Our studies indicate that several connection subdomain and RNase H domain substitutions typically act as pre-therapy polymorphisms.

1. Introduction

The zidovudine (AZT)-resistance mutations reside at the DNA polymerase domain of HIV-1 reverse transcriptase (RT). They are associated either with (a) the exclusion mechanism that enhances discrimination at the point of AZT monophosphate (AZT-MP) incorporation through a set of mutations at codons A62, V75, F77, F116 and Q151 of the polymerase domain (Deval et al., 2002; Ueno and Mitsuya, 1997), or with (b) the excision mechanism that involves selective removal of AZT-MP after it has been incorporated by RT into the viral DNA (Boyer et al., 2001; Meyer et al., 1999). The excision mechanism is associated with mutations at the polymerase domain, including M41L, D67N, K70R, L210W, T215F/Y and K219E/Q (excision-containing mutations [EEMs] also known as thymidine analogue-associated mutations [TAMs]).

Certain mutations in the connection subdomain (CD; codons 322–440) of the polymerase domain or in the RNase H domain (codons 441–560) of HIV-1 RT have recently been shown to be associated with resistance to AZT (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Ntemgwa et al., 2007; Yap et al., 2007). In some cases it appears that mutations that affect AZT resistance have different phenotypes, depending on the presence or absence of other resistance mutations. For example, the polymorphism G333D/E does not confer drug resistance by itself, but has been reported to contribute significantly to dual AZT-lamivudine (3TC) resistance when combined with EEMs and M184V (Caride et al., 2000; Gallego et al., 2002; Kemp et al., 1998; Zelina

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et al., 2008). Similarly, A371V and Q509L, which were selected in the background of D67N and K70R by high concentrations of AZT *in vitro*, show strong resistance to AZT and weak cross-resistance to 3TC, abacavir (ABC) and tenofovir (TNF/PMPA) in the presence of EEMs (Brehm et al., 2007). Santos et al. (2008) also recently reported that the A360V and A371V mutations are frequently observed in AZT-treated patients. In contrast, one of the connection subdomain mutations, N348I, is associated with resistance to both nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) and appears to be induced by regimens containing AZT, didanosine (ddI) and/or nevirapine (NVP) (Hachiya et al., 2008; Yap et al., 2007). Recently, it has been shown that the N348I mutation decreases the efficiency of RNase H cleavage and increases excision of AZT from AZT-terminated primer/templates, in the presence of the pyrophosphate donor ATP (Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008; Yap et al., 2007). The decreased degradation of the RNA template by the diminished RNase H cleavage has been proposed to provide additional time for RT to excise AZT-MP and hence result in the observed increased AZT resistance (Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008).

With the exception of N348I, the clinical relevance of these mutations remains to be clarified. A major obstacle to understanding the contribution of connection subdomain mutations to NRTI or NNRTI resistance has been the shortage of relevant sequencing data. This is because, until recently, the majority of commercially available genotypic and phenotypic assays have not been targeting this region of the enzyme. This is now changing, as more attention is being focused on such substitutions, following recent publications from us (Hachiya et al., 2008) and others (Yap et al., 2007) showing that at least one connection subdomain mutation, N348I, contributes to multi-class drug resistance. However, it has not yet been determined if the genotypic substitutions encountered in the connection subdomain of polymerase or in the RNase H domain of RT have any phenotypic impact or any effect on virologic response to subsequent therapies. Another important question is whether resistance testing now performed should include these mutations.

To ascertain whether some mutations at the connection subdomain or at the RNase H domain of RT that appear in the absence of known drug-resistance mutations of the polymerase domain are induced by reverse transcriptase inhibitor (RTI) treatment or are simply pre-existing polymorphisms, we determined the frequency of amino acid substitutions in antiretroviral treatment-naïve patients and assessed whether these substitutions at the reported sites (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) can cause drug resistance by themselves. We also explored whether these substitutions may have any effect on the virologic response to subsequent therapies.

2. Materials and methods

2.1. Patients

A total of 123 clinical isolates were obtained from fresh plasma of treatment-naïve HIV-infected patients using MAGIC-5 cells as described previously (Hachiya et al., 2001). Written informed consent was obtained from each patient under approval by the Institutional Review Board of the International Medical Center of Japan (IMCJ-H13-80). The clinical course and antiretroviral therapies used were reviewed retrospectively.

2.2. Recombinant molecular clones

Recombinant molecular clones were generated as described previously (Hachiya et al., 2008). Briefly, the pBS-RT_{WT} contains almost entire RT coding sequence (amino acid position 14–560) containing silent mutations for cloning (restriction enzyme sites, Xma I and Xba I at 5′- and 3′-end of DNA fragment, respectively). After site directed mutagenesis, the mutated RT was ligated into the corresponding restriction enzyme site of the HIV infectious clone pNL101 (Hachiya et al., 2008; Shimura et al., 2008).

2.3. Genotypic and phenotypic assays

For the genotypic assay, viral RNA was extracted from the culture supernatant of clinical isolates, amplified by nested RT-PCR, and then directly sequenced as described previously (Hachiya et al., 2008). For subtype classification, the RT sequences were analyzed using the 'Genotyping' software (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) which uses the BLAST algorithm. HIV-1 sequences in worldwide, treatment-naïve patients were obtained from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/index.html, accessed as late as 26 February 2008) and compared with our cohort. Prevalence of mutations at each codon were compared by the χ^2-test, or Fisher's exact test when the number of patients was smaller than 5.

For phenotypic assay, each clinical isolate was directly tested for drug susceptibility in triplicates, using the MAGIC-5 cell-based assay as described previously (Hachiya et al., 2001). Infectious viruses were obtained by transfection of 293T cells with individual HIV molecular clones containing the desired mutations that were introduced by site directed mutagenesis. Cells were subsequently harvested and examined with the MAGIC-5 cell based assay (Hachiya et al., 2001, 2008).

2.4. Measurements of HIV-1 viral load

To assess virologic outcome, HIV-1 viral loads in plasma were measured using the commercially available Amplicor HIV-1 Monitor Test (Version 1.5, Roche Diagnostics K.K., Basel, Switzerland). Mean change from 0 at weeks 4, 8, 12, 16, 20 and 24 were evaluated. The statistical significance of the longitudinal changes of HIV-1 viral load in plasma was assessed by the Mann–Whitney *U*-test.

2.5. Molecular modeling studies

The SYBYL and O programs were used to prepare molecular model of the complexes of HIV-1 RT in complex with RNA/DNA (Protein Data Bank code number 1HYS), and containing mutations A376S, N348I and Q509L that were introduced manually into the original 1HYS structure. After introduction of the mutations, the structure coordinates were optimized through 100 cycles of Coleman energy minimization protocol.

3. Results

3.1. Sequence analysis of RT region

We sequenced nearly the entire RT coding region (amino acid position 9–560) of 123 clinical isolates obtained from treatment-naïve patients. Among these isolates, six contained the known RTI-associated resistant mutations, D67N ($n=2$), K238S ($n=2$) (http://www.hiv.lanl.gov/content/index), V108I/K238S ($n=1$) and V106A/V108I/K238S ($n=1$), and thus were excluded from further analysis. The clinical isolates were obtained from six patients within 1 year of the diagnoses for HIV-1 infection. Prevalence of HIV-1 with drug-associated mutations in Japanese treatment-naïve patients is estimated at approximately 4% (Gatanaga et al., 2007) and in American and European patients at 8–27% (Descamps et al., 2005; Little et al., 2002; UK Collaborative Group on Monitoring the Transmission

of HIV Drug Resistance, 2001; Weinstock et al., 2004). Therefore, the prevalence in our cohort (4.8%) seems to be comparable or lower than in previous reports, suggesting that the six patients are treatment-naïve and newly infected from treated patients. The strong majority of the remaining samples in our cohort were of subtype B ($n = 101$ of a total of 117 isolates), while other subtypes were also identified (CRF01_AE, A, C and CRF12_BF, with12, 2, 1 and 1 isolates, respectively).

Substitutions at the connection subdomain and RNase H domain observed in this cohort and in previous reports (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) are shown in Table 1. In the treatment-naïve patients of our cohort that were infected with subtype B ($n = 101$), the frequencies of all mutations associated with AZT-resistance (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) were comparable to those (treatment-naïve) deposited in the Stanford HIV Drug Resistance Database, except for the A360T mutation. The G335D and A371V substitutions were more prevalent in the non-B, rather than in the B isolates of our cohort. Moreover, the G335D/A371V combination was observed in 9 (56.3%) of the non-B isolates. Other polymorphisms, including E312A/D/N/T, G335E/N/S, A360S/T, A371T, A376T/V and Q509H, were widely observed in all subtypes in our cohort as well as in the Stanford HIV Drug Resistance Database. None of the clinical isolates of our cohort had the G333D, G335C, N348I, A360I/V, and Q509L mutations.

3.2. Phenotypic assay for clinical isolates

Phenotypically, all clinical isolates showed little resistance to tested drugs (Table 1). Isolates with the V365I substitution ($n = 4$ in subtype B) showed slightly reduced susceptibility to 3TC (2.3-fold). However, V365I may not be clinically relevant, since generally at least 3-fold resistance is required for assigning 3TC resistance *in vivo* (Parkin et al., 2004; Rhee et al., 2006). Furthermore, the prevalence of V365I in treated and untreated patients in the Stanford HIV Drug Resistance Database is comparable (3.7% and 3.6%, respectively). Notably, clinical isolates from treatment-naïve patients from our cohort with HIV carrying the E312N, G335E/N or A376V substitutions displayed rather enhanced susceptibility (over five-fold) to

Table 1
Drug susceptibilities of 117 clinical isolates obtained from *treatment-naïve patients*.

Amino acid substitutions	Frequency[a] % (n)		Median fold change in resistance[b]			
	Subtype B ($n = 101$)	Non-B ($n = 16$)	AZT	3TC	NVP	EFV
E312	84.1 (85)	18.8 (3)	1.2	1.3	1	1.1
Q[c]	**3 (3)**	**0**	**1.3**	**1.4**	**1.2**	**1.1**
A	6.9 (7)	0	1.1	1.1	1.3	1
D	1 (1)	6.3 (1)	1.7	1.2	1.7	1.1
N	0	6.3 (1)	0.1	1.3	0.2	1.2
T	5 (5)	68.8 (11)[d]	0.8	1	1.1	1.1
G333	94.1 (95)	100 (16)	1.1	1.3	1.1	1.1
D[c]	**0**	**0**	**–**	**–**	**–**	**–**
E[c]	**5.9 (6)**	**0**	**1.4**	**1.5**	**1**	**1.4**
G335	95 (96)	25 (4)	1.2	1.4	1	1.1
C[c]	**0**	**0**	**–**	**–**	**–**	**–**
D[c]	**2 (2)**	**68.8 (11)**[d]	**0.7**	**0.9**	**1.1**	**1.1**
E	0	6.3 (1)	0.3	0.06	0.2	0.5
N	1 (1)	0	0.2	0.2	0.8	1.5
S	2 (2)	0	0.6	0.9	1.3	1.4
N348	100 (101)	100 (16)	1.1	1.3	1	1.1
I[c,e]	**0**	**0**	**–**	**–**	**–**	**–**
A360	79.2 (80)	87.5 (14)	1.1	1.3	1.1	1.1
I[c]	**0**	**0**	**–**	**–**	**–**	**–**
V[c]	**0**	**0**	**–**	**–**	**–**	**–**
S	0	6.3 (1)	0.7	1.2	1.5	0.8
T	20.8 (21)[f]	6.3 (1)	0.9	1.4	1	1.2
V365	96 (97)	100 (16)	1.1	1.2	1	1.1
I[c]	**4 (4)**	**0**	**1**	**2.3**	**1.7**	**1.3**
A371	96 (97)	25 (4)	1.2	1.3	1.1	1.1
V[c]	**3 (3)**	**75** (12)[d]	**0.7**	**0.9**	**0.9**	**1.1**
T	1 (1)	0	0.5	1.3	0.7	0.7
A376	92.1 (93)	75 (12)	1.1	1.3	1	1.1
S[c]	**3 (3)**	**6.3 (1)**	**1.3**	**0.9**	**1**	**0.6**
T	5 (5)	12.5 (2)	1.2	1	1.4	1.2
V	0	6.3 (1)	0.1	1.3	0.2	1.2
Q509	98 (99)	100 (16)	1.1	1.3	1.1	1.1
L[c]	**0**	**0**	**–**	**–**	**–**	**–**
H	2 (2)	0	0.6	0.7	0.7	0.8

[a] Of 123 clinical isolates, six carried the known RTI-associated mutations and were excluded from this analysis.

[b] The drug susceptibility assay (Hachiya et al., 2001) was clinically accepted in Japan.

[c] Resistant mutations reported previously (Brehm et al., 2007; Delviks-Frankenberry et al., 2007, 2008; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Ntemgwa et al., 2007; Santos et al., 2008; Yap et al., 2007) are indicated in bold. Greater than three-fold increase of EC_{50} compared to that of NL4-3 was defined as resistance.

[d] The prevalence of these substitutions (E312T, G335D and 371V) is significantly difference among treatment-naïve patients between subtype B and non-B isolates ($p < 0.0001$).

[e] N348I confers cross-resistance to NRTIs and NNRTIs (Hachiya et al., 2008; Yap et al., 2007).

[f] The prevalence of A360T is significantly higher in our cohort compared to the Stanford HIV Drug Resistance Database (8.7%, $p = 0.0021$).

Table 2
Drug susceptibilities of molecular HIV-1 clones.

Mutation	EC_{50}, μM (fold increase)[a]				
	NRTI			NNRTI	
	AZT	3TC	TNF[b]	NVP	EFV
WT	0.026 ± 0.009	0.42 ± 0.04	6.2 ± 1.5	0.023 ± 0.01	0.0012 ± 0.0001
E312Q	0.037 ± 0.006 (1.4)	0.36 ± 0.05 (0.9)	4.1 ± 1.4 (0.7)	0.056 ± 0.007 (2.4)	0.0009 ± 0.0002 (0.8)
G333D	0.04 ± 0.01 (1.5)	0.28 ± 0.1 (0.7)	4.5 ± 1.8 (0.7)	0.055 ± 0.01 (2.4)	0.0017 ± 0.0003 (1.4)
G335C	0.04 ± 0.02 (1.5)	0.45 ± 0.1 (1.1)	7.7 ± 1.1 (1.2)	0.065 ± 0.02 (2.8)	0.0007 ± 0.00009 (0.6)
N348I	0.14 ± **0.01 (5.4)**	0.56 ± 0.07 (1.3)	8.8 ± 1.9 (1.4)	0.24 ± **0.04 (10)**	0.0032 ± 0.0005 (2.7)
A360I	0.037 ± 0.01 (1.4)	0.35 ± 0.1 (0.8)	7.1 ± 2.1 (1.1)	0.038 ± 0.01 (1.7)	0.0009 ± 0.00008 (0.8)
A360V	0.03 ± 0.002 (1.2)	0.28 ± 0.09 (0.7)	5.7 ± 2.3 (0.9)	0.051 ± 0.01 (2.2)	0.0016 ± 0.0006 (1.3)
V365I	0.045 ± 0.008 (1.7)	0.27 ± 0.06 (0.6)	6.1 ± 2.0 (1)	0.066 ± 0.02 (2.9)	0.0013 ± 0.0002 (1.1)
A376S	0.053 ± 0.02 (2)	0.3 ± 0.03 (0.7)	5.9 ± 1.6 (1)	0.084 ± **0.02 (3.7)**	0.0022 ± 0.0004 (1.8)
Q509L	0.072 ± 0.02 (2.8)	0.45 ± 0.1 (1.1)	8.1 ± 2.7 (1.3)	0.21 ± **0.06 (9.1)**	0.0032 ± 0.0009 (2.7)

[a] Data means ± standard deviations from at least three independent experiments. The relative increase in the EC_{50} value compared with that in HIV-1_{WT} is given in parentheses. Bold indicates an increase in EC_{50} value greater than three-fold.
[b] TNF (PMPA) [(*R*)-9-(2-phosphonomethoxypropyl) adenine or tenofovir] is the active nucleotide of the clinical prodrug tenofovir disoproxil fumarate.

AZT and NVP, AZT, 3TC and NVP, and AZT and NVP, respectively (Table 1). In our cohort, in the absence of EEM mutations, A371V had no significant effect on drug resistance (Table 1). However, other studies have shown that combined with EEMs, A371V can confer strong resistance to AZT and A371V has also been recently reported to be associated with weak cross-resistance to 3TC, TNF/PMPA and ABC (Brehm et al., 2007). In our cohort, ABC inhibits efficiently the clinical isolates that contain the A371V substitution in the absence of EEMs ($n = 13$) either in a subtype B, or non-B background (median fold increase was 0.9-fold, data not shown). Further, the combination of A371V and G335D commonly observed in non-B isolates also showed no resistance to AZT, 3TC or ABC (0.7-, 1.0- and 1.1-fold increase in susceptibility as compared to wild-type HIV, respectively). These results demonstrate that none of the above substitutions that were observed in clinical isolates confer any significant resistance to NRTIs or NNRTIs in the absence of EEMs.

3.3. Phenotypic assay for molecular clones

To further expand our understanding of the role of substitutions in these RT regions on drug resistance we also prepared HIV-1 recombinant viruses with related mutations that have been reported previously in similar drug resistance studies (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007). The results shown in Table 2 confirm that in the absence of NRTI or NNRTI resistance mutations, most substitutions in the connection subdomain and RNase H domain (with the exception of N348I, A376S and Q509L) show no significant resistance to AZT, 3TC, TNF/PMPA, NVP or efavirenz (EFV) (less than three-fold), suggesting that these mutations act as secondary mutations and may enhance resistance that is caused by primary mutations and/or may somehow improve replication kinetics impaired by the primary mutations. Q509L, which has been reported to enhance

Table 3
Profiles of patients infected with HIV carrying connection subdomain substitutions, and initial therapies used in patient treatments.

Parameter	Combination for treatment-naïve patients infected HIV-1			
	With substitutions		Without substitutions	
	With AZT ($n = 8$)	Without AZT ($n = 13$)	With AZT ($n = 16$)	Without AZT ($n = 24$)
Male, *n* (%)	5(63)	10(77)	15(94)	23(96)
Median age (range)	37 (27–60)	41 (27–54)	36 (24–55)	38 (26–59)
Median baseline viral load, log_{10} copies/ml (range)	5.0 (3.0–6.0)	5.0 (4.2–5.8)	5.0 (4.1–6.4)	5.2 (4.2–6.3)
Median baseline CD4 cell count, cell/μl (range)	217 (3–549)	110 (3–332)	225 (9–613)	170 (4–760)
Substitutions in the connection subdomain, *n* (%)[a]				
E312Q	–	3(23)	–	–
G333E	2(25)	2(15)	–	–
G335D	3(38)	6(46)	–	–
V365I	2(25)	–	–	–
A371V	2(25)	5(38)	–	–
A376S	1(13)[b]	2(15)	–	–
Initial therapy, *n* (%)				
Zidovudine	8(100)	–	16(100)	–
Lamivudine	4(50)	11(85)	12(75)	24(100)
Stavudine	–	11(85)	–	20(83)
Didanosine	4(50)	–	4(25)	–
Abacavir	1(13)	1(8)	1(6)	3(13)
Tenofovir	–	1(8)	–	1(4)
Emtricitabine	–	1(8)	–	–
Nevirapine	–	–	–	3(13)
Efavirenz	2(25)	3(23)	9(56)	9(38)
One protease inhibitor (PI)	3(38)	7(54)	5(31)	7(29)
Dual-boosted PI	1(13)	2(15)	1(6)	5(21)

[a] E312Q, G333E, G335D, V365I, A371V and V376S were reported to be AZT-resistant mutations (Brehm et al., 2007; Kemp et al., 1998; Nikolenko et al., 2007).
[b] In this case, the viral load did not fall below the limits of detection.

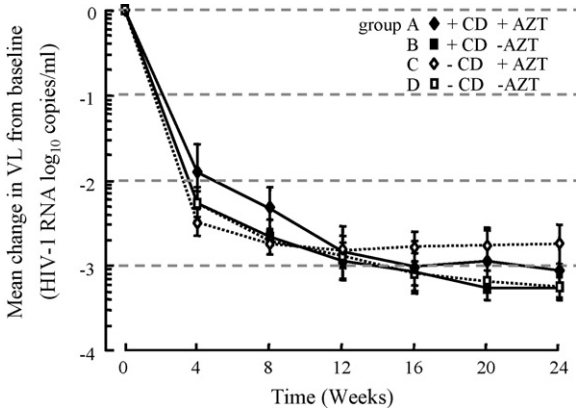

Fig. 1. Virological response up to 24 weeks after initiation of combination therapy. Mean (±standard error of the mean; S.E.M.) changes in plasma viral load (VL) were measured by Amplicor HIV-1 Monitor Test (Version 1.5, Roche Diagnostics K.K., Basel, Switzerland) from 0 to 24 weeks. Treatment-naive patients that were subsequently treated with combination therapy regimens are classified into four groups: patients that were infected with HIV-1 containing connection subdomain (CD) mutations and that subsequently received either combination therapy with AZT ($n = 8$, closed diamonds, group A) or without AZT ($n = 13$, closed squares, group B) and patients who were infected with HIV-1 with none of connection subdomain substitutions, and who subsequently received combination therapy with either AZT ($n = 16$, open diamonds with broken line, group C) or without AZT ($n = 24$, open squares with broken line, group D).

cross resistance to NRTIs in the presence of EEMs (Brehm et al., 2007), conferred little resistance to at least AZT, 3TC and TNF/PMPA in this study. Unlike N348I that confers dual resistance to NRTIs and NNRTIs, A376S and Q509L provided only NVP resistance.

3.4. Virological response after initiation of combination therapy

To further assess whether the CD substitutions at baseline are one of predictive factors of virologic outcome, we examined clinical samples from the treatment-naïve patients who subsequently received combination therapy through measuring virus load in plasma from 0 to 24 weeks (Table 3 and Fig. 1). The treatment-naïve patients were classified in four groups: (A) patients who were infected by HIV-1 that carried one or two of the CD substitutions E312Q, G333E, G335D, V365I, A371V or A376S and who subsequently received combination therapy that contained AZT ($n = 8$); (B) patients who were infected by HIV-1 that carried the above CD substitutions and who subsequently received combination therapy that did not contain AZT ($n = 13$); (C) patients who were infected by HIV-1 that did not carry any of the above CD substitutions and who subsequently received combination therapy containing AZT ($n = 16$); and (D) patients who were infected by HIV-1 that did not carry the above CD substitutions and who subsequently received combination therapy that did not contain AZT ($n = 24$). The mean change in viral load from baseline (week 0) to week 24 was from -2.76 to -3.28 $\log_{10}$ copies/ml among four groups. There were no significant differences in viral load changes up to 24 weeks among these groups (Fig. 1). Marginal viral suppression was observed in one patient who was infected by HIV-1 carrying A376S and who received combination therapy containing AZT. Any of the drug-associated resistant mutations were detected during the first 5 months of receiving combination therapy. However, HIV-1 protease mutations D30N and M36I that are responsible for resistance to NFV and HIV-1 RT D67N mutation that is responsible for AZT resistance eventually emerged. After switching to a new combination regimen (d4T/3TC/LPV), the viral load was successfully decreased. These results indicate that at least combination of two substitutions in the connection subdomain that are observed in treatment-naïve patients do not affect the virologic response of the ensuing combination therapy. Instead, they merely act as polymorphisms among the treatment-naïve patients.

4. Discussion

According to the crystal structure of HIV-1 RT in complex with RNA/DNA, some amino acids in the connection subdomain may affect binding to the RNA/DNA substrate (Sarafianos et al., 2001). It has been proposed that mutations at the connection subdomain may alter the binding affinity for nucleic acid at the connection subdomain and lead to enhanced resistance to AZT when combined with EEMs. This is thought to happen through a decrease in template RNA degradation which in turn provides additional time for RT to excise AZT-MP from the AZT-terminated template-primer$_{AZT\text{-}MP}$, thus causing resistance to AZT (Delviks-Frankenberry et al., 2007; Nikolenko et al., 2005, 2007). In our cohort, as well as in the Stanford HIV Drug Resistance Database, we observed a considerable number of treatment-naïve clinical samples containing substitutions (E312Q, G333E, G335D, V365I, A371V and A376S) that have been previously associated with AZT resistance. Our phenotypic studies with clinical isolates carrying mutations located in the connection subdomain of the polymerase or in the RNase H domain of RT revealed that in the absence of other known NRTI or NNRTI resistance mutations they do not cause by themselves significant resistance to the tested RTIs. Additionally, results from our cohort establish that the presence of G333E, G335D, V365I or A371V among treatment-naïve patients does not play any significant role in the virologic response after initiation of therapies that may, or may not, include AZT. We identified 25 isolates that have been deposited before 1986, prior to clinical trails for AZT in the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov/content/index). Some of these isolates also contained E312V, V365I, A376S/T/P, indicating that at least these substitutions are polymorphisms that preceded any antiviral therapy.

None of the isolates in our cohort had the H539N or H549N substitutions which have been proposed to be associated with resistance to NRTIs due to decreasing the frequency of RT template-switching and the level of RNase H activity (Nikolenko et al., 2004; Roquebert and Marcelin, 2008). Furthermore, the G333D, G335C, N348I, A360I/V and Q509L substitutions were not observed in our cohort, and were also rarely observed among treatment-naïve patients (less than 1%) in the Stanford HIV Drug Resistance Database. Their increased incidence among NRTI-treated patients as compared to untreated patients (>3-fold, >40-fold and >12-fold increases for G333C, N348I, and A360V respectively [http://hivdb.stanford.edu/cgi-bin/RTPosMutSummary.cgi]) and in the case of Q509L reported by others (Brehm et al., 2007; Roquebert et al., 2007) suggests that they are associated with AZT resistance. However, site directed mutagenesis studies showed that G333D (Kemp et al., 1998), G335C (Nikolenko et al., 2007), A360I/V (Nikolenko et al., 2007) and Q509L (Brehm et al., 2007) did not confer significant AZT resistance in the absence of other AZT resistance mutations. At present, only N348I has been shown to be involved in resistance to multiple RTIs (Hachiya et al., 2008; Yap et al., 2007). HIV with a serine at codon 376 also exhibits some NVP resistance in the absence of other mutations (Table 2). However, clinical isolates harboring different residues at position 376 exhibited no significant changes in their drug susceptibilities (Table 1). This discrepancy may arise from strain-specific polymorphisms that are present in the clinical isolates or the reference virus used in this study that may influence NVP susceptibility positively or negatively, respectively. In fact, we observe several polymorphisms in the majority of these isolates and it is possible that they somehow affect drug resistance. For instance, V118I has been identified in 2% of treatment-naïve patients as one of strain-specific polymorphisms, but more frequently observed in RTI-treated patients

(Delaugerre et al., 2001). Although this mutation by itself confers no resistance, it has been reported to contribute to hypersusceptibility to NNRTI (Clark et al., 2006) as well as resistance to NRTI in the presence of E44A/D and/or EEMs (Romano et al., 2002). Therefore, it is possible that polymorphisms present in our clinical isolates may also affect drug-susceptibility leading to minor discrepancies with the results obtained with recombinant virus.

In this study, the reference clone has an A376T polymorphism that is observed in a wide range of subtypes. Therefore, it is unlikely that A376T affects NVP susceptibility. Q509L confers moderate (~9-fold) resistance to NVP (Table 2). Although Q509L was not observed in our cohort, this mutation was found in the pretreated patients of another survey ($n = 118$) (Roquebert et al., 2007). These results indicate that introduction of Q509L may alter virologic responses, especially for NVP, although so far the clinical relevance and virological response of Q509L among the antiretroviral-experienced patients remains to be elucidated by further experiments.

Analysis of the crystal structure of RT bound to RNA/DNA showed that residues 376 (of the p66 subunit) and 509 are located relatively close to the nucleic-acid binding cleft of RT, and residue 348 of the p66 subunit is located close to the hinge region of the thumb subdomain and to the NNRTI-binding pocket (Fig. 2). Recently, Abbondanzieri et al. demonstrated that binding of nevirapine to RT causes conformational changes to the enzyme, allowing it to somehow relax the grip on nucleic-acid substrate (Abbondanzieri et al., 2008; Arnold and Sarafianos, 2008). NVP acts as a rapid-equilibrium inhibitor, not a tight-binding inhibitor as EFV (Maga et al., 2000; Motakis and Parniak, 2002), and it might be more sensitive to changes in the interaction between RT and the nucleic acid substrate. Thus, changes in the interactions of RT with nucleic-acid substrate could also influence the interaction balance between polymerase and RNase H activity and consequently might lead to RTI resistance. Nevertheless, additional biochemical and structural studies are warranted to define the exact mechanisms by which these mutations in the connection subdomain and RNase H domains confer NVP resistance.

Several studies have reported a correlation between two distinct types of EEMs in various HIV subtypes (Kantor et al., 2005; Montes et al., 2004; Novitsky et al., 2007). The Type I EEMs (M41L, L210W, T215Y and occasionally the D67N mutation) appear twice as frequently as Type II EEMs (D67N, K70R, T215F and K219Q mutation) in subtype B (Marcelin et al., 2004), whereas Type II EEMs are mostly observed in non-B isolates (Montes et al., 2004; Novitsky et al., 2007). Type II EEMs confer lower levels of AZT and TNF/PMPA resistance, as compared to Type I EEMs (Cozzi-Lepri et al., 2005; Miller et al., 2004). Addition of A371V to Type II EEM background conferred cross-resistance to AZT and tenofovir (Brehm et al., 2007). A371V was observed in the majority of non-B isolates in our cohort (75%) and the Stanford HIV Drug Resistance Database (96% in CRF01_AE). Therefore, it is possible that in the background of non-B isolates, the majority of which contains drug resistance associated connection subdomain mutations, smaller number of EEMs, especially Type II EEMs, might be preferentially selected for AZT and TNF/PMPA resistance. In the absence of EEMs, mutations at the connection subdomain of non-subtype B HIV, such as E312N, G335E or A376 V, appear to act as simple polymorphisms, because they either maintain or enhance drug susceptibility in non-subtypes B HIV (Table 1). However, the A376S polymorphism in samples of treatment-naive patients or in a recombinant virus used in this study conferred mild NVP resistance (Table 2). These mutations were stable even in the absence of any drug treatment, suggesting that viral fitness of these variants is likely to be comparable to wild type non-subtype B HIV.

In this study we report the prevalence of amino acid substitutions in the connection subdomain of the polymerase domain and in the RNase H domain of RT in a cohort of treatment-naïve patients. We also determined the phenotypic susceptibility of these mutants to various RTIs. Our results support the hypothesis that the substitutions observed among treatment-naïve patients have little impact on therapeutic outcome by themselves in the absence of AZT-associated mutations, although certain substitutions, such as N348I, A376S, and Q509L, are involved in drug resistance even by themselves. These results may help improve existing interpretation algorithms and analysis of drug resistance mutations.

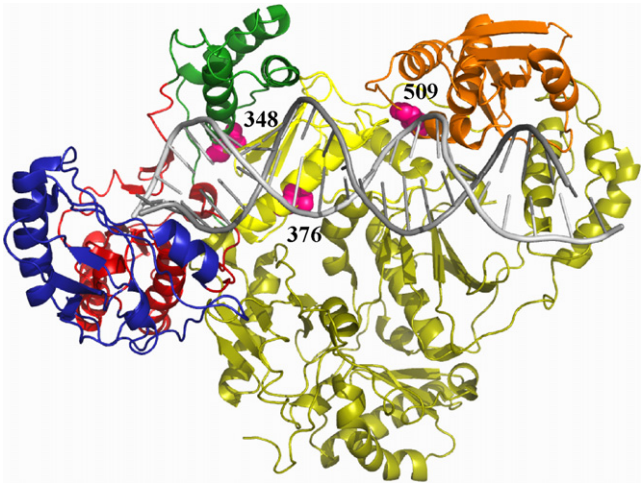

Fig. 2. Structure of HIV-1 RT in complex with RNA/DNA. The fingers, palm, thumb, connection subdomains, and RNase H domain of the p66 subunit colored in blue, red, green, yellow and orange, respectively. The p51 subunit is shown in dark yellow. Residue 348 of the p66 subunit is shown as pink Van der Waals spheres, and located proximally to the hinge region of the thumb subdomain and to the NNRTI binding pocket. Residues 376 and 509 of the p66 subunit are also shown, and are located proximally to the nucleic acid binding cleft.

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