

A376S in the Connection Subdomain of HIV-1 Reverse Transcriptase Confers Increased Risk of Virological Failure to Nevirapine Therapy

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Background. The clinical relevance of mutations in the connection subdomain and the ribonuclease (RNase) H domain of HIV-1 reverse transcriptase (RT) is uncertain.

Methods. The risk of virological failure to nonnucleoside RT inhibitor (NNRTI)-based antiretroviral therapy (ART) was evaluated in NNRTI-naïve patients who started NNRTIs in the EuroSIDA study after July 1997 according to preexisting substitutions in the connection subdomain and the RNase H domain of HIV-1 RT. An observed association between A376S and virological failure was further investigated by testing in vitro NNRTI susceptibility of single site-directed mutants and patient-derived recombinant viruses. Enzymatic assays also determined the effects of A376S on nevirapine and template-primer binding to HIV-1 RT.

Results. Virological failure occurred in 142 of 287 (49%) individuals: 77 receiving nevirapine (67%) and 65 receiving efavirenz (38%) ($P < .001$). Preexisting A376S was associated with an increased risk of virological failure to nevirapine (relative hazard [RH] = 10.4; 95% confidence interval [CI], 2.0–54.7), but it did not affect efavirenz outcome the same way (RH = 0.5; 95% CI, 0.1–2.2) (P value for interaction = .013). A376S conferred selective low-level nevirapine resistance in vitro, and led to greater affinity for double-stranded DNA.

Conclusions. The A376S substitution in the connection subdomain of HIV-1 RT causes selective nevirapine resistance and confers an increased risk of virological failure to nevirapine-based ART.

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Despite the clinical success of potent antiretroviral therapy (ART), the development of human immunodeficiency virus type 1 (HIV-1) drug resistance still constitutes a major hurdle for the long-term efficacy of current regimens. Several evolutionary pathways lead to resistance, including mutations in reverse transcriptase (RT) selected under the pressure of nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). However, certain mutations or polymorphisms in the RT connection subdomain and ribonuclease (RNase) H domain may also modulate HIV-1 susceptibility to RT inhibitors in vitro [1–9]. Such mutations are often coselected on the same genome alongside thymidine analogue resistance-associated mutations (TAMs) and are more prevalent in subjects treated with NRTIs than in antiretroviral-naïve individuals. When combined with TAMs, connection subdomain mutations

increase resistance to azidothymidine by reducing template RNA degradation and enhancing azidothymidine excision [6, 8]. To a lesser extent, they also increase cross-resistance to lamivudine, abacavir, and tenofovir, but not to stavudine or didanosine. At least 3 mutations in the connection subdomain (N348I, T369I, and E399G/D) reduce HIV-1 susceptibility to NNRTIs, possibly by affecting dimerization of p66/p51 heterodimers [9–12]. However, the impact of such mutations on the outcome of antiretroviral treatment remains largely unknown [1, 13].

Prospective, randomized clinical trials have shown comparable clinical outcomes of first-line ART including nevirapine or efavirenz in antiretroviral-naïve HIV-1–infected subjects [14, 15]. In contrast, observational comparative studies have consistently reported inferior virological and immunological outcomes of nevirapine-based regimens relative to efavirenz [16–20].

We investigated the association between the presence of a predefined set of mutations in the connection subdomain and RNase H domain of RT and the subsequent risk of virological failure to a first NNRTI-based antiretroviral therapy. Moreover, we explored the viral phenotypic implications and biochemical mechanisms involved in this association.

METHODS

Subjects

This study included NNRTI-naïve individuals enrolled in the EuroSIDA (a pan-European observational study) cohorts who started NNRTI-based triple ART after July 1997 with known pretherapy HIV-1 RNA and CD4⁺ counts, at least 2 HIV-1 RNA measures after initiation of ART, and a plasma sample available within 1 year before initiation of NNRTI-based treatment (ie, baseline sample).

Genotyping

Population sequencing of HIV-1 protease (PR) and RT was performed using the Trugene HIV-1 Sequencing Kit (Siemens Medical Solutions). The connection subdomain (RT codons 315–423) and the RNase H domain (RT codons 424–560) were sequenced using an in-house method (Supplementary data). The GenBank accession numbers for the HIV-1 polymerase sequences used in this analysis were from HQ684850 to HQ685120. Resistance mutations in PR and RT were defined according to the International AIDS Society (IAS)–USA classification [21]. Twelve predefined substitutions in the connection and RNase H domains (G335C/D, N348I, A360I/V, V365I, T369I, A371V, A376S, E399G/D, and Q509L) [1, 2, 4, 6–9] were identified using the HIVdb Program [22].

Association Between Mutations in the Connection Subdomain and the RNase H Domain and Virological Failure

Virological failure was defined as 2 consecutive HIV-1 RNA determinations above 500 copies/mL (at least 6 months after initiation of NNRTI therapy if baseline HIV-1 RNA was above

500 copies/mL). The date of virological failure corresponded to the first of these 2 measurements. Time to virological failure was evaluated using Cox regression models, stratified by clinical center and adjusted for previous use of ART and AIDS diagnosis, year of initiation of NNRTI, CD4⁺ T-cell count at baseline, nadir CD4⁺ T-cell count, plasma HIV-1 RNA at baseline, maximum plasma HIV-1 RNA, number of non-NNRTI-active drugs in the regimen, and predicted NNRTI susceptibility (the latter 2 estimated using Rega software, version 7.1), with follow-up time right censored at the time of the penultimate available viral load measurement. A separate model for each of the 12 candidate connection and RNase H mutations was fitted, and Wald tests were used to examine the significance of the interaction with the specific NNRTI used. *P* values <0.05 were taken to be statistically significant except in the case of multiple comparisons where a Bonferroni correction was used to account for the inflated type I error.

Mutational Covariation Analysis

Covariation between connection and RNase H mutations and IAS-USA resistance mutations in RT was evaluated using the phi correlation statistic implemented in the “R” software package [23]. The *P* values were adjusted using the Benjamini-Hochberg method.

Generation of Site-Directed Mutant Viruses

K103N (a mutation commonly associated with resistance to nevirapine and efavirenz) and A376S were generated into HIV strain NL4-3 (HIV_{NL4-3}) by site-directed mutagenesis [24, 25] (Supplementary data).

Generation of Patient-Derived Recombinant Clones

Nine subjects with the A376S substitution and a random sample of 9 of those who did not harbor the A376S substitution were identified from the parent study cohort. Both groups were balanced in terms of age, CD4⁺ T cell count, plasma viral load, and number of drugs received; subjects had no NNRTI resistance and only limited NRTI genotypic resistance. Recombinant viruses containing patient-derived Gag-PR-RT segments (HXB2 positions 1811–4335) were produced by cotransfection with the plasmid pJM31ΔGPRT in MT-4 cells [24]. Sequence homology with plasma viral population-based sequences was verified on the newly generated recombinant viruses.

Phenotypic Assays

Susceptibility of recombinant viruses to efavirenz (Bristol-Myers Squibb), nevirapine (Boehringer-Ingelheim), etravirine (National Institutes of Health AIDS Research and Reference Reagent Program), and delavirdine (Enzo Life Sciences) was tested as described previously [26]. The fold change in drug susceptibility was determined by dividing the median inhibitory concentration (IC₅₀) of every sample virus by the IC₅₀ of the drug-susceptible HIV-1_{NL4-3} (Supplementary data).

RT Expression and Purification

The RT coding sequences of wild-type viral clones (HIV-1_{NL4-3}) and mutants K103N and A376S were polymerase chain reaction amplified as described elsewhere [27]. Purified DNA was then cleaved with EcoRI and XhoI and cloned into the expression vector pRT66B(BH10) [28]. Recombinant heterodimeric p66/p51 HIV-1 RTs were expressed and purified as previously described [27–29]. The number of RT active sites was determined by active-site titration [30] using the DNA-DNA template primer D38/25PGA [28, 31].

Enzymatic Assays

RT inhibition assays were carried out with template primer D38/25PGA (30 nM) and purified RT in 7.5 mM HEPES, pH 7.0, containing 3.75 mM sodium chloride, 3.75 mM magnesium acetate, 130 mM KCH₃COO, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol, and 1% dimethyl sulfoxide. RT and template primer were preincubated for 10 min at 37°C and, after an additional incubation with the corresponding NNRTI at different concentrations (10 min, 37°C), polymerization reactions were initiated by adding 2'-deoxythymidine 5'-triphosphate (dTTP) (25 μM, final concentration). The IC₅₀ for each enzyme and NNRTI was obtained after analyzing the reaction products using denaturing polyacrylamide gel electrophoresis. The equilibrium dissociation constants (K_d) for nevirapine binding to binary complexes of RT and DNA-DNA (D38/25PGA) were determined in the buffer mentioned above as described elsewhere [32, 33]. The equilibrium dissociation constants for wild-type and mutant HIV-1 RTs and DNA-DNA duplexes (ie, D38/25PGA) were determined as previously described [34] in 25 mM HEPES, pH 7.0, containing 15 mM sodium chloride, 15 mM magnesium acetate, 130 mM KCH₃COO, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol, 1% (v/v) dimethyl sulfoxide, and 25 μM dTTP (Supplementary data).

RESULTS

Subjects

Sequence data were obtained for 287 patients; 115 (40.1%) started treatment with nevirapine and 172 (59.9%) with efavirenz (Table 1). Relative to those initiating efavirenz therapy, subjects starting with nevirapine were less likely to be ART-naive, initiated NNRTI therapy earlier, had greater previous exposure to NRTIs, and initiated protease inhibitor (PI) therapy concurrently with NNRTIs more often.

Prevalence of Resistance Substitutions at the Initiation of NNRTI Therapy

Mutations in the connection and RNase H domains of HIV-1 RT were equally distributed among subjects starting nevirapine and efavirenz ($P = .185$) (Figure 1), thus ruling out that pre-existing differences in the prevalence of these mutations could explain the difference in risk of virological failure between

efavirenz and nevirapine. The most frequent mutations in the connection subdomain of HIV-1 RT were: A371V (23%), G335D (14%), and A376S (9%). The M184V mutation in RT was also equally distributed between subjects initiating nevirapine (57%) or efavirenz (56%). At least 1 TAM was detected in 68% of subjects initiating therapy with nevirapine, and in 58% of individuals starting with efavirenz (data not shown).

Covariation Between Substitutions in the Connection Subdomain and RNase H Domain and RT Inhibitor Resistance-Associated Mutations

On the basis of the Benjamini-Hochberg-adjusted P values, mutation V365I was weakly associated with L210W ($\Phi = .23$; $P = .02$), mutation A371V was associated with M41L ($\Phi = .21$; $P = .02$), and mutation E399D was associated with the multidrug resistance 151M cluster ($\Phi = .32$, $P = .04$). These associations were also found in subjects with prior exposure to zidovudine or stavudine ($n = 231$), of whom 10 (4%) had the V365I and L210W mutations ($P = .22$); 41 (18%) had the A371V and M41L mutations ($P < .001$), and 3 (1%) had the E399D and 151M mutations ($P = 1.0$). Thus, only the cluster A371V/M41L appeared to occur more frequently than was expected by chance alone.

Preexisting Substitutions in the Connection Subdomain and the RNase H Domain of HIV-1 RT and Risk of Virological Failure

A total of 142 (49%) subjects experienced virological failure over a median of 19 months of follow-up: 77 started therapy with nevirapine (67%) and 65 with efavirenz (38%) ($P < .001$). The multivariable model adjusted for multiple potentially confounding factors (including mutations detected in RT) showed an increased risk of virological failure in subjects starting treatment with nevirapine versus efavirenz (relative hazard [RH] = 2.0; 95% confidence interval [CI], 1.2–3.3; $P = .006$). The effects of harboring specific mutations in the connection subdomain and the RNase H domain of HIV-1 RT were then assessed both overall and according to NNRTI used (Table 2). Given the need for multiple comparisons, a Bonferroni correction was used to adjust the level of significance and P values $< .005$ were taken as statistically significant. None of the mutations in the connection subdomain or the RNase H domain of HIV-1 RT showed an independent association with the virological response to NNRTI-based regimens. A376S was the only substitution showing a markedly different association with the outcome comparing patients starting nevirapine-based regimens (RH = 10.4; 95% CI, 2.0–54.7) with those who started efavirenz-including combinations (RH = 0.5; 95% CI, 0.1–2.2). The interaction between the presence of the A376S mutation and the specific NNRTI started was found to be significant ($P = .013$); this indicates that the predictive value of A376S is likely to be different according to the NNRTI used. Similar results were obtained after excluding anti-retroviral-naive subjects (data not shown), those with IAS-USA NNRTI-associated mutations, and those with D67N, K70R, or T215F mutations (Figure 1).

Table 1. Patient Characteristics at the Start of the NNRTI-Containing Regimen

	Total	Nevirapine	Efavirenz	P value
All (n, %)	287 (100.0%)	115 (40.1%)	172 (59.9%)	...
Date started regimen (Median, IQR)	Nov00 (Feb99–Feb03)	Apr99 (Jun98–Jun01)	Oct01 (Mar00–Jan04)	<.001
Demographics (n, %)				
Male	210 (73.2%)	83 (72.2%)	127 (73.8%)	.755
HIV exposure (n, %)				
Homosexual	138 (48.1%)	55 (47.8%)	83 (48.3%)	...
IDU	53 (18.5%)	23 (20.0%)	30 (17.4%)	...
Heterosexual	72 (25.1%)	24 (20.9%)	48 (27.9%)	...
Other	24 (8.4%)	13 (11.3%)	11 (6.4%)	...
Previous AIDS (n, %)	86 (30.0%)	31 (27.0%)	55 (32.0%)	.363
Subtype (n, %)				
B	248 (86.4%)	100 (87.0%)	148 (86.0%)	...
Non-B	39 (13.6%)	15 (13.0%)	24 (14.0%)	...
Age (years) (Median, IQR)	41 (35–47)	41 (34–47)	41 (35–46)	.875
CD4 count (cells/mm ³) (Median, IQR)				
Baseline	240 (160–340)	210 (144–320)	252 (171–362)	.052
Nadir	125 (44–209)	98 (35–210)	140 (48–208)	.310
Viral load (log ₁₀ copies/mL) (Median, IQR)				
Baseline	4.5 (3.8–5)	4.4 (3.8–5)	4.5 (3.8–5)	.403
Maximum ever	5.1 (4.7–5.6)	5.1 (4.7–5.5)	5.1 (4.8–5.6)	.391
Antiretroviral use at start of regimen (n, %)				
Antiretroviral-naive	47 (16.4%)	10 (8.7%)	37 (21.5%)	.004
No. NRTIs previously used				
0	47 (16.4%)	10 (8.7%)	37 (21.5%)	...
1–2	48 (16.7%)	24 (20.9%)	24 (14.0%)	...
3–4	141 (49.1%)	57 (49.6%)	84 (48.8%)	...
≥5	51 (17.8%)	24 (20.9%)	27 (15.7%)	...
No. PIs previously used				
0	86 (30.0%)	31 (27.0%)	55 (32.0%)	...
1	55 (19.2%)	30 (26.1%)	25 (14.5%)	...
≥2	146 (50.9%)	54 (47.0%)	92 (53.5%)	...
Antiretrovirals in regimen (n, %)				
Total no. of drugs in regimen				
3–4	239 (83.3%)	98 (85.2%)	141 (82.0%)	...
≥5	48 (16.7%)	17 (14.8%)	31 (18.0%)	...
NRTIs in regimen				
≤1	50 (17.4%)	21 (18.3%)	29 (16.9%)	...
2	197 (68.6%)	85 (73.9%)	112 (65.1%)	...
≥3	40 (13.9%)	9 (7.8%)	31 (18.0%)	...
PIs in regimen				
0	166 (57.8%)	60 (52.2%)	106 (61.6%)	...
1	63 (22.0%)	35 (30.4%)	28 (16.3%)	...
≥2	58 (20.2%)	20 (17.4%)	38 (22.1%)	...

NOTE. HIV, human immunodeficiency virus; IDU, intravenous drug user; IQR, interquartile range; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

Drug Susceptibility of Single Site-Directed Mutants

The single-mutant virus containing A376S showed a 4.3-fold increase in nevirapine resistance in cell culture assays, but remained susceptible to efavirenz, etravirine, and delavirdine (≤2.5-fold change) in comparison with the reference HIV-1_{NL4-3} strain (Figure 2). On the other hand, the K103N mutant

virus showed high-level resistance to nevirapine and significant resistance to efavirenz and delavirdine, while remaining susceptible to etravirine (Figure 2) [35].

Drug Susceptibility of Patient-Derived Recombinant Viruses

Recombinant viruses containing the 3'-end of Gag, protease, and the RT region derived from 17 patients in whom no primary

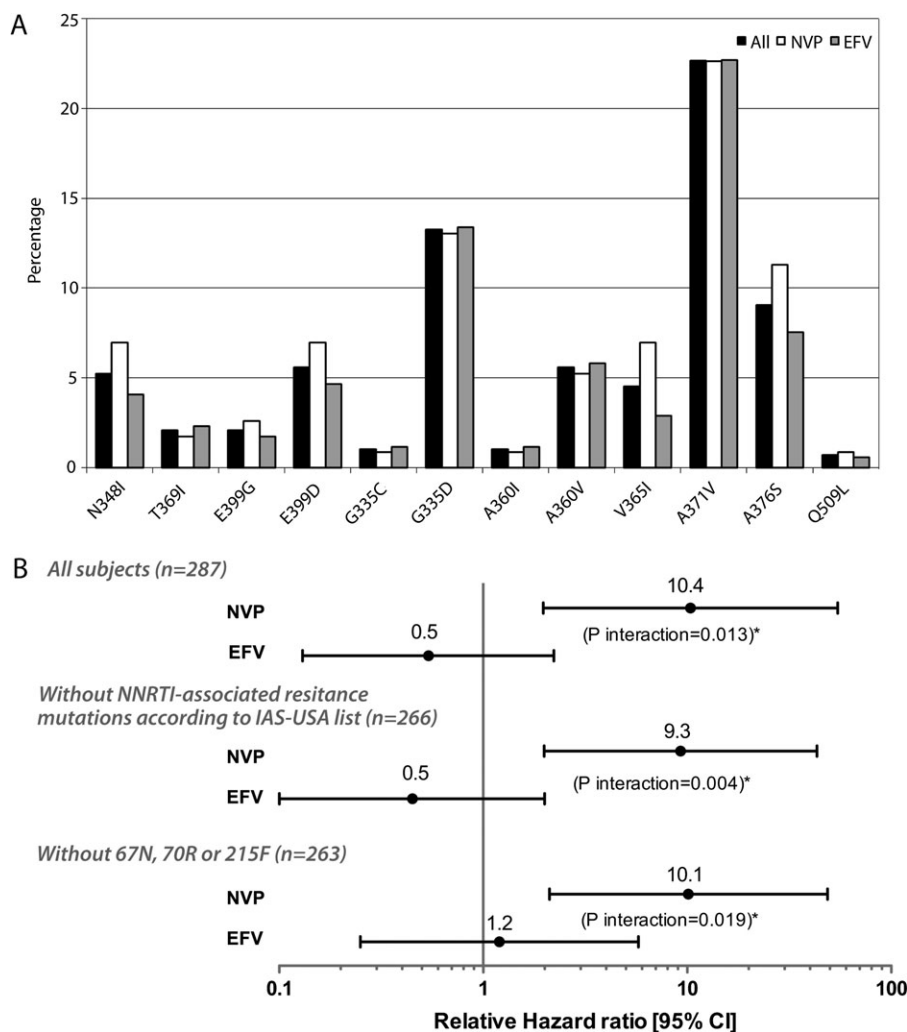


Figure 1. Prevalence of preexisting substitutions in the connection and ribonuclease (RNase) H domains of HIV-1 reverse transcriptase (RT) at the initiation of nonnucleoside RT inhibitor (NNRTI) therapy and risk of virological failure. (A) Mutations in the connection subdomain and RNase H domain detected at initiation of the NNRTI-containing regimen. NVP, nevirapine; EFV, efavirenz. (B) Relative risk of virological failure associated with the detection of the A376S mutation according to whether patients started nevirapine or efavirenz overall and in the subgroups of patients with International AIDS Society–USA (IAS-USA) NNRTI-associated mutations and those with D67N, K70R, or T215F mutations. *Wald t -test for the interaction term calculated as the product of the 2 binary variables, one indicating whether A376S was detected and the other indicating which NNRTI was started. CI, confidence interval.

NNRTI mutations were detected were generated to evaluate the putative nevirapine resistance induced by the A376S mutation. Eight were obtained from subjects with A376S HIV-1 mutants and 9 from subjects with viruses lacking this substitution and previously balanced in terms of age, CD4⁺ T-cell count, plasma viral load, and number of drugs received. One A376S recombinant virus did not grow in vitro. Recombinant viruses containing the A376S substitution showed a median 5.5-fold decrease in nevirapine susceptibility; this contrasts with the 2.2-fold change observed in viruses lacking the substitution ($P = .046$; Mann–Whitney test) (Figure 2).

Inhibition of Wild-Type and Mutant RTs

The IC₅₀ of nevirapine, delavirdine, efavirenz, and etravirine was determined for recombinant wild-type HIV-1_{NL4-3} RT and for

K103N, and A376S (Table 3). As expected, K103N conferred resistance to nevirapine, delavirdine, and efavirenz and had no effect on susceptibility to etravirine. Efavirenz and etravirine were found to be the most effective inhibitors for wild-type RT under our assay conditions, whereas nevirapine was less potent than the other NNRTIs. The A376S substitution conferred <2-fold resistance to all inhibitors except for nevirapine (3.8-fold change); this result is consistent with those of the previous experiments carried out with recombinant viruses (Figures 2 and 3).

Effects of A376S on Nevirapine-Binding Affinity

The role of A376S in nevirapine resistance was further confirmed by comparing the binding affinities of the inhibitor to RT/DNA–DNA binary complexes containing wild-type and

Table 2. Relative Hazard Ratios of Virological Failure After Starting NNRTI-Containing Antiretroviral Therapy

Mutation	No.	Univariate analysis			Multivariate analysis ^a		
		RH	95% CI	<i>P</i> value	RH	95% CI	<i>P</i> value
A. Overall							
N348I	15	1.7	.8–3.5	.187	1.0	.5–2.3	.942
T369I	6	1.4	.5–3.9	.569	1.7	.5–6.2	.444
E399G	6	1.1	.3–3.9	.884	0.8	.1–5.2	.843
E399D	16	2.2	1.0–4.6	.046	1.7	.8–3.7	.190
G335C	3	1.1	.2–4.9	.946	1.2	.2–6.3	.848
G335D	38	0.9	.5–1.5	.663	0.9	.5–1.7	.805
A360I	3	0.9	.1–6.5	.894	1.6	.2–12.6	.639
A360V	16	0.8	.4–1.9	.712	0.9	.4–2.0	.735
V365I	13	1.8	.8–4.0	.174	1.8	.7–4.6	.237
A371V	65	1.9	1.2–2.8	.002	1.7	1.1–2.7	.026
A376S	26	1.4	.7–2.8	.280	1.0	.5–2.1	.987
Q509L	2
B. Patients starting nevirapine							
N348I	8	1.1	.4–3.1	.861	1.0	.3–3.1	.994
T369I	2	1.2	.2–9.5	.850	6.8	.7–65.6	.098
E399G	3	2.5	.5–13.1	.277	2.1	.2–28.3	.586
E399D	8	1.9	.6–5.6	.241	1.7	.5–5.6	.392
G335C	1	1.1	.1–12.7	.917	0.8	.1–10.8	.864
G335D	15	1.2	.6–2.5	.616	0.8	.4–1.8	.595
A360I	1	2.0	.2–16.0	.516	11.1	.9–134	.058
A360V	6	0.5	.1–1.7	.266	0.9	.2–4.0	.848
V365I	8	2.1	.6–6.5	.224	0.6	.1–3.1	.538
A371V	26	2.9	1.5–5.6	.002	2.8	1.2–6.4	.013
A376S	13	2.6	.9–7.2	.072	10.4	2.0–54.7	.006
Q509L	1
C. Patients starting efavirenz							
N348I	7	1.7	.5–5.6	.412	1.1	.3–4.7	.892
T369I	4	2.0	.5–8.4	.325	2.6	.2–30.6	.448
E399G	3
E399D	8	2.1	.7–6.9	.201	1.8	.6–6.1	.318
G335C	2	1.7	.2–12.6	.645	2.5	.3–21.9	.394
G335D	23	0.4	.1–1.0	.044	0.4	.1–1.4	.143
A360I	2
A360V	10	1.0	.4–3.0	.954	0.9	.3–3.0	.874
V365I	5	0.9	.1–7.0	.916	1.6	.2–14.1	.649
A371V	39	1.4	.8–2.6	.271	1.6	.7–3.5	.244
A376S	13	0.7	.2–2.5	.625	0.5	.1–2.2	.389
Q509L	1

NOTE. Given the need for multiple comparisons, a Bonferroni corrected level of significance was used and *P* values <.005 were taken to be statistically significant. CI, confidence interval; NNRTI, nonnucleoside reverse transcriptase inhibitor; RH, relative hazard.

^a Multivariate analyses adjusted for number of drugs taken previously (nucleoside reverse transcriptase inhibitors and protease inhibitors), previous AIDS diagnoses, year NNRTI therapy was started, CD4 count (baseline, nadir), viral load (baseline, maximum), number of non-NNRTI-active drugs in regimen, and NNRTI baseline resistance (both defined by Rega software version 7.1), and for Table 2A, whether patients started nevirapine or efavirenz therapy.

A376S RTs. Pre-steady-state kinetics were used to measure the burst amplitude of nucleotide incorporation (ie, RT bound to

DNA-DNA in the first turnover) in the presence of increasing concentrations of nevirapine. We found that A376S produced a 2.8-fold reduction in the affinity for the inhibitor. Average K_d values obtained from 3–4 independent experiments were $5.88 \pm 1.12 \mu\text{M}$ for wild-type HIV-1_{NL4-3} RT and $16.64 \pm 3.43 \mu\text{M}$ for mutant A376S (Figure 3).

Effects of A376S on Template-Primer Binding Affinity

Both wild-type and mutant A376S RTs have a similar kinetic mechanism, where fast nucleotide incorporation is followed by a slow steady-state release of the duplex DNA from the enzyme. Because catalysis is much faster than DNA release, the burst amplitude in single nucleotide incorporation is a direct measurement of the amount of RT/DNA-DNA binary complex proceeding to the first turnover. It also facilitates determination of the equilibrium dissociation constant of DNA from wild-type and mutant RTs. Figure 3 shows the dependence of burst amplitude (ie, bound D38/25PGA complex) on the total (bound and free) DNA concentration for the wild-type enzyme and A376S. Based on this approach, we determined K_d values of $3.07 \pm 0.71 \text{ nM}$ for wild-type HIV-1_{NL4-3} RT (average of 6 independent experiments) and $1.63 \pm 0.29 \text{ nM}$ for mutant A376S (average of 8 independent experiments).

Structural Studies

Residue 376 is polymorphic in the RT of group-M-subtype-B HIV-1 RT, with >60% of viral isolates from naive patients harboring Ala, 24% from patients harbouring Thr, and 6% from patients harboring Ser (Stanford HIV Drug Resistance Database; <http://hivdb.stanford.edu>). All of the Protein Data Bank coordinates for HIV-1 RT crystal structure coordinates contain Thr at position 376, because the enzymes derive from the HXB2 strain of HIV-1 (see Supplementary methods for details). Thr376 occupies an internal position in p51, whereas in p66 it lies near the p66/p51 dimerization interface (Figure 4). In HIV-1 RT complexes containing nevirapine, delavirdine, efavirenz, or etravirine, the distance between the amino acid at position 376 and the NNRTI binding pocket was >20 Å for the p66 residue and >40 Å for the same position in p51. Therefore, low-level resistance to nevirapine cannot be attributed to a direct effect of A376S in the structure of the NNRTI binding pocket. In addition, Thr376 is also far from the nucleic acid binding cleft (Figure 4). However, Thr376 (in p66) could affect interactions between p66 and p51, because the neighboring residues Gln373 of p66 and Trp401 of p51 are important contributors to the heterodimer interface [36] (Figure 4). Interatomic distances of 4.2 Å between the side chain of Thr376 and the side chain of Trp401 of p51 have been measured in the ternary complex of HIV-1 RT/DNA-DNA/dTTP. However, in binary complexes of HIV-1 RT and nucleic acids (DNA-DNA and RNA-DNA duplexes), these distances are reduced to <4 Å. The presence of Ala376 instead of Ser is expected to weaken these interactions.

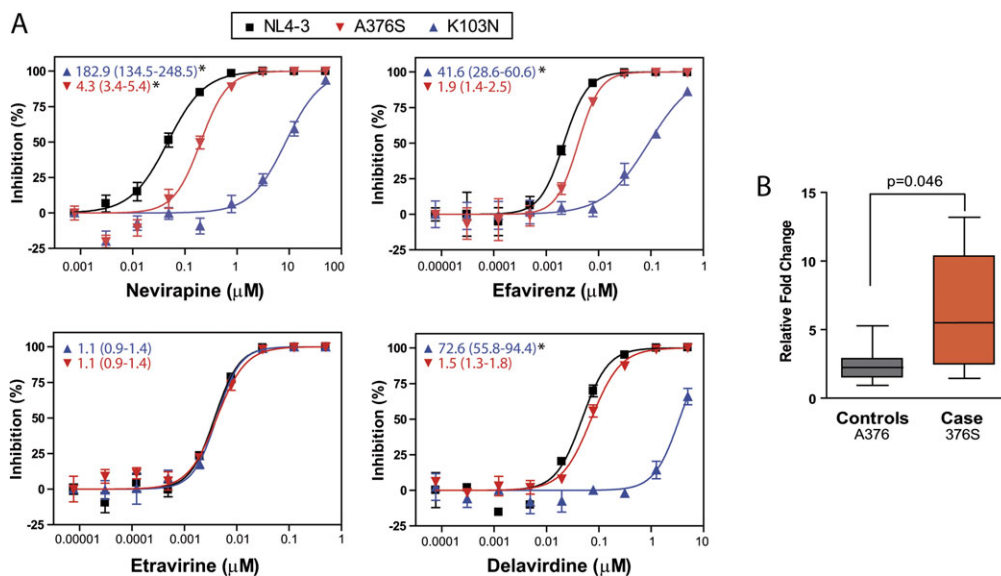


Figure 2. In vitro nonnucleoside reverse transcriptase inhibitor (NNRTI) susceptibility of single site-directed mutants and patient-derived recombinant viruses. (A) Inhibition of infection in the presence of increasing concentrations of different NNRTIs: Single-mutant A376S viruses and K103N viruses were tested in parallel with the wild-type human immunodeficiency virus type 1 strain NL4-3 (HIV-1_{NL4-3}) in TZM-bl cell cultures. *Fold change values above the cutoff (2.5-fold) for each drug. (B) Relative fold change resistance to nevirapine of recombinant viruses derived from plasma viral RNA from NNRTI-naive patients: Viruses from case patients harbor the A376S mutation in the connection subdomain ($n = 8$), whereas the control cases contain the wild-type alanine amino acid in this position ($n = 9$). All viruses were tested in parallel and the fold change in drug susceptibility was determined by dividing the median inhibitory concentration (IC₅₀) of every sample virus by the IC₅₀ of the drug-susceptible HIV-1_{NL4-3}. A nonparametric Mann–Whitney test was used to analyze differences between groups.

DISCUSSION

This study found that NNRTI-naive individuals infected with viruses carrying the A376S substitution were 10-fold more likely to experience virological failure if they initiated nevirapine-based ART. Phenotypic assays on site-directed mutants, patient-derived recombinant viral constructs, and recombinant RT enzymes confirmed a selective low-level decrease in nevirapine susceptibility in the presence of the A376S substitution, as well as a greater affinity for double-stranded DNA in the mutant enzyme than in the wild-type enzyme. Remarkably, the A376S substitution did not seem to have a large effect on the virological outcome of efavirenz-based regimens, nor did it affect the phenotypic susceptibility of the virus to efavirenz, delavirdine, or etravirine.

NNRTIs act as chemical enhancers of HIV-1 RT dimerization [37, 38]. Efavirenz has been shown to be the most potent enhancer of RT heterodimerization, whereas nevirapine had a weak effect and delavirdine had no effect [37]. Structurally, such a stabilizing effect is restricted to the vicinity of the NNRTI binding site. One possible interpretation of our biochemical findings is that A376S confers higher dimerization capacity to HIV-1 RT. The higher affinity for the template primer observed in our RT/DNA-DNA binding assays could be the result of a higher dimerization capacity, since more nevirapine is required to disrupt RT/DNA-DNA binding. Indeed, structural inference suggests that A376S could favor hydrophobic interactions between the side chains of Trp401 and Ser/Thr376, leading to p66/p51 heterodimer stabilization. The fact that delavirdine did not show this behavior could be due to a slightly

Table 3. Inhibition of Wild-Type and Mutant HIV-1 RT by NNRTIs

RTs	IC ₅₀ (μM), Mean ± Standard Deviation ^a			
	Nevirapine	Efavirenz	Etravirine	Delavirdine
Wild-type	12.2 ± 4.2	0.72 ± 0.09	0.56 ± 0.11	4.79 ± 0.48
K103N	>1600 (>130)	26.6 ± 1.6 (36.9)	1.02 ± 0.09 (1.8)	>1600 (>330)
A376S	45.9 ± 7.8 (3.8)	1.03 ± 0.02 (1.4)	1.06 ± 0.12 (1.9)	8.41 ± 1.2 (1.8)

NOTE. HIV-1, human immunodeficiency virus type 1; IC₅₀, median inhibitory concentration; NNRTI, nonnucleoside reverse transcriptase inhibitor; RT, reverse transcriptase.

^a Each of the assays was performed independently at least 3 times. The fold increase in IC₅₀ relative to the wild-type NL4-3 RT is shown in parentheses.

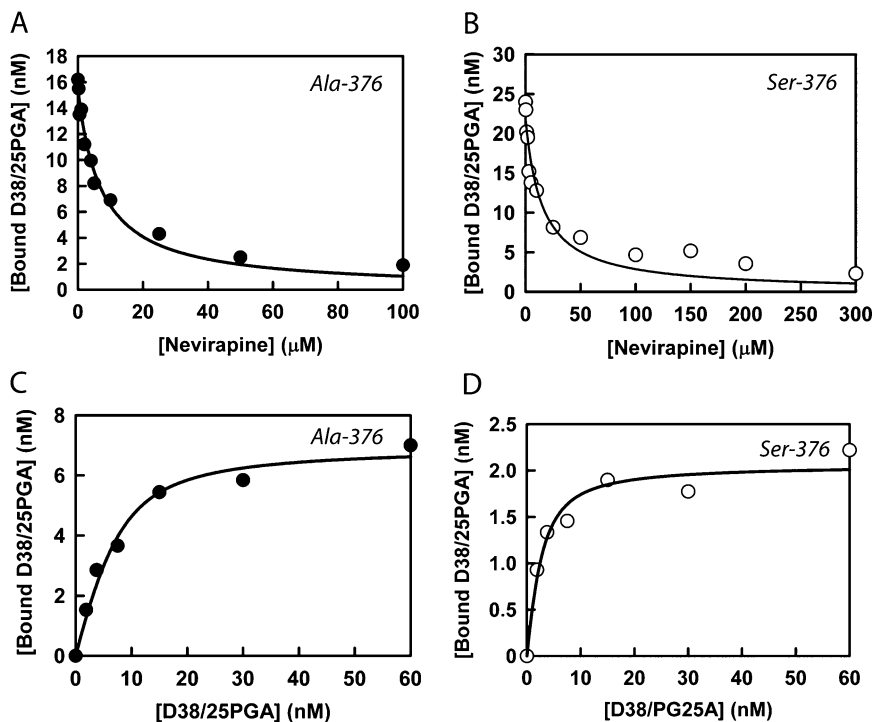


Figure 3. Effects of the A376S substitution on nevirapine and template-primer binding to human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). *A* and *B*, Determination of the equilibrium constant of nevirapine for RT bound to D38/25PGA. Burst amplitudes were plotted against nevirapine concentration and fitted to a hyperbolic function (solid line) to give K_d values of $7.18 \pm 0.94 \mu\text{M}$ for wild-type HIV-1 strain NL4-3 (HIV_{NL4-3}) RT (*A*) and $15.4 \pm 3.71 \mu\text{M}$ for mutant A376S (*B*). *C* and *D*, DNA-DNA template-primer binding affinities of wild-type HIV-1_{NL4-3} RT and mutant A376S. RTs and increasing concentrations of 5'-³²P-labeled 25/38-mer were incubated for 10 minutes at 37°C in reaction buffer containing 15 mM magnesium acetate. The RT/DN-DNA complex was rapidly mixed with 25 μM 2'-deoxythymidine 5'-triphosphate and quenched with EDTA-formamide. Burst amplitudes (bound RT/DNA-DNA) were plotted as a function of the total DNA concentration and fitted to a quadratic equation (solid line) to obtain K_d values of $2.69 \pm 0.79 \text{ nM}$ for wild-type HIV-1_{NL4-3} RT (*C*) and $1.53 \pm 0.51 \text{ nM}$ for mutant A376S (*D*).

increased affinity of A376S for the template primer, or simply to the lack of ability to stabilize the heterodimer. Unlike in the case of N348I [39], the decreased nevirapine susceptibility conferred by A376S does not seem to be related to effects on primer removal during initiation of (+)-strand DNA synthesis (data not shown). Previous studies associated A376S with increased zidovudine resistance [6]. However, our analysis of the risk of virological failure in patients receiving nevirapine comparing those harboring or not harboring A376S was adjusted for the predicted activity of nucleosides. Other authors have also reported that A376S confers 3.7-fold increased resistance to nevirapine [1].

Previous studies explored the role of connection subdomain and RNase H domain mutations emerging during treatment failure with RT inhibitors. Mutations N348I or T369I decreased NNRTI susceptibility in vitro [12, 40], and the appearance of N348I was associated with a concomitant increase in viral load in patients failing RT inhibitor-based regimens [12, 40]. However, none of these studies assessed the impact of preexisting mutations in treatment response. Our analysis shows that none of the preexisting connection subdomain or RNase H domain mutations (including A376S) showed an association with

response to an NNRTI-based regimen after Bonferroni correction (Table 2). However, A376S clearly showed a differential effect on virological outcome according to whether efavirenz or nevirapine were used in the regimen.

To appreciate the clinical implications of our findings, it is important to note that A376S was found in 9% of all NNRTI-naive subjects in our cohort. A similar prevalence of 6%–7% in NNRTI-naive patients infected with HIV-1 subtype B is currently reported in an independent database (Stanford HIV Drug Resistance Database; <http://hivdb.stanford.edu>) [12, 40]. According to these estimations, approximately 6%–9% of NNRTI-naive subjects harboring mutation A376S have a 10-fold higher risk of virological failure than those not carrying this mutation if they are started with a nevirapine-containing regimen. This might not be a major clinical problem at present, because nevirapine is mostly being used as an alternative to efavirenz for initial therapy. However, nevirapine has been shown to be noninferior to atazanavir as first-line therapy, has the most favorable lipid profile of all NNRTIs and PIs [41–43], is the cheapest antiretroviral drug, and might become available soon as a generic drug in several European countries. These factors could modify prescription patterns for initial therapy in

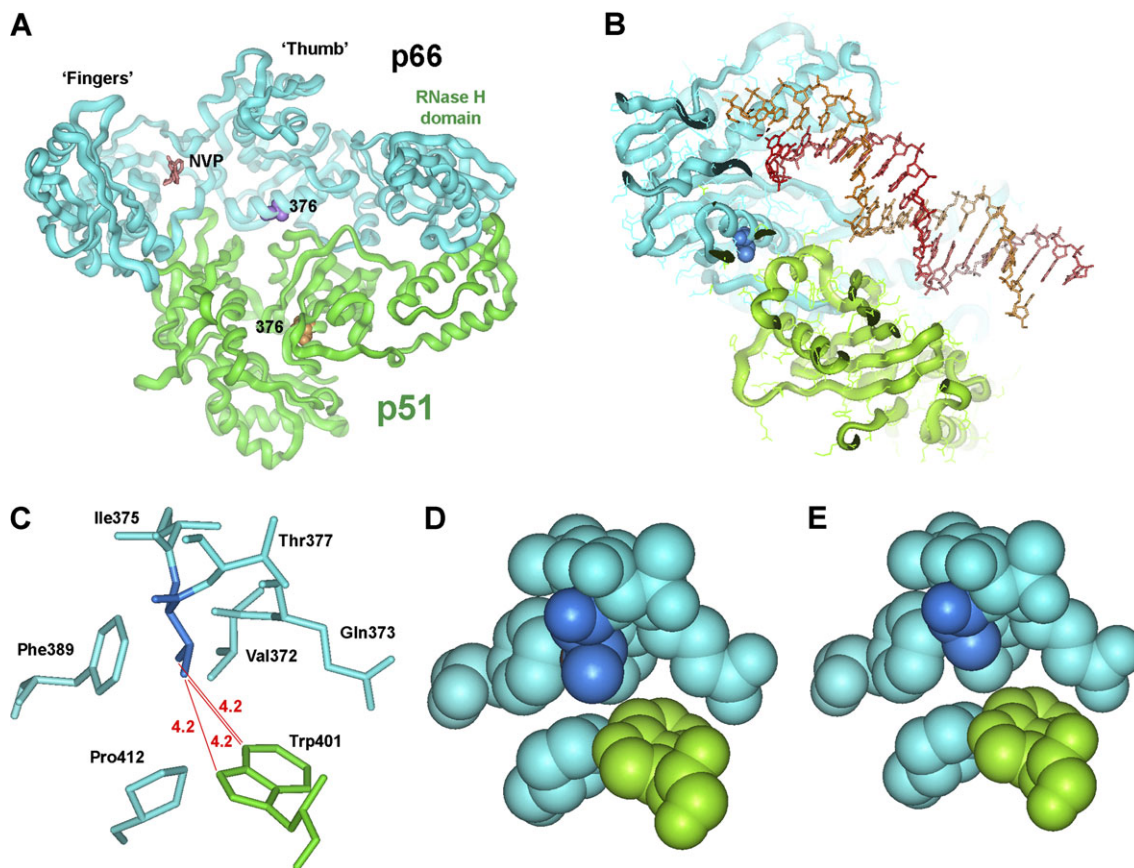


Figure 4. Structural location of Thr376 of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). *A*, Crystal structure of HIV-1 RT bound to nevirapine showing the location of position 376 in p66 (blue ribbon) and p51 (green ribbon). *B*, Location of Thr376 of p66 (dark blue space-filling model) relative to the DNA-DNA template primer in the ternary complex of HIV-1 RT containing a DNA template primer and 2'-deoxythymidine 5'-triphosphate (dTTP). *C*, Amino acid residues in the vicinity of Thr376 of p66, in the ternary complex of HIV-1 RT/DNA-DNA/dTTP. The residues shown are located at $<5 \text{ \AA}$ from Thr376 (dark blue). Residues in p66 are shown in light blue and Trp401 of p51 is shown in green. Relevant interatomic distances are shown in red. *(D)* and *(E)* are space-filling models of the interactions represented in *(C)*. *(E)* shows a model where Thr376 has been replaced by Ala. The relative positions of residues *(C-E)* correspond to the RT orientation shown in *(B)*.

Europe. In any event, the clinical relevance of A376S genotyping in antiretroviral-naïve individuals living in resource-rich settings will parallel the level at which nevirapine is prescribed as initial therapy. As of today, it might prove useful to rule out the presence of A376S in antiretroviral-naïve candidates before prescribing nevirapine.

A376S genotyping could also be potentially useful in resource-limited settings, where nevirapine is currently the initial “third” drug of choice, provided that suitable alternatives for initial treatment are available and economically viable. However, it is important to note that our study population was predominantly infected with a subtype B virus, and that all phenotypic and mechanistic studies were performed in subtype B backgrounds. Therefore, our data cannot be used to directly infer the effect of this substitution in non-B subtypes.

Finally, A376S genotyping is unlikely to be useful in treatment-experienced subjects. First-generation NNRTIs are hardly ever included in second or further treatment lines, because their

low genetic barrier confers an increased risk of virological failure in the presence of resistance to other drugs in the regimen. Furthermore, A376S does not significantly affect susceptibility to etravirine, and did not largely affect the virological response in the DUET trials [44]. Therefore, A376S genotyping does not add value to predicting responses to etravirine.

The main limitations of our study are its relatively small sample size and the unbalanced distribution of several key baseline characteristics between the treatment groups. A larger sample would be needed to decrease the uncertainty surrounding our relative hazard estimates. We have adjusted for measured factors that could have confounded the association between A376S and virological outcome in our multivariable analysis, but we cannot rule out other unmeasured or unknown confounders (eg, adherence, prescription patterns) that could affect our results.

In conclusion, the A376S substitution in the RT connection subdomain alone confers selective nevirapine resistance by

increasing the affinity of RT for double-stranded DNA, which results in >10-fold increased risk of virological failure to nevirapine-based ART in NRTI-experienced subjects. This same magnitude of increase in risk could be ruled out in patients who started efavirenz-based regimens. According to the local prevalence of A376S, genotyping of the connection subdomain might be useful to tailor treatment in antiretroviral-naïve HIV-1-infected subjects considering NNRTIs as first-line therapy.

Supplementary Data

Supplementary data are available at the *Journal of Infectious Diseases* online.

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