Poster 646

CROI 2009

Mutation A376S in the RT Connection Domain Is Associated with an Increased Risk of Virological Failure to Nevirapine-Based Therapy in NNRTI-Naïve HIV-Infected Subjects in the EuroSIDA Study

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INTRODUCTION

Large-scale, prospective, randomised clinical trials demonstrate comparable clinical outcomes of first-line antiretroviral treatment (ART) with nevirapine (NVP) or efavirenz (EFV) in ART-naïve HIV-1-infected subjects.[1] Conversely, observational comparative studies [2-6] consistently report inferior virological and immunological outcomes of NVP-based regimens relative to EFV. One possible explanation for such discrepancy among others is that observational studies usually include NNRTI-naïve but NRTI-experienced subjects. These subjects are more likely to be infected with HIV containing mutations in the reverse transcriptase (RT) connection (RT-C) or RNAseH (RT-H) domains, which could modulate the virological outcomes of NNRTI-based ART.

Mutations in the RT-C and RT-H domains are coselected on the same genome as TAMs. RT-C mutations increase zidovudine resistance when combined with TAMs [7-9] by reducing template RNA degradation and enhancing AZT excision;[10] to a lesser extent, they also increase cross-resistance to lamivudine, abacavir, and tenofovir.[9] Importantly, three RT-C mutations (N348I, T369I, and E399G/D) also confer reduced NNRTI susceptibility *in vitro*, possibly by affecting dimerization of p66/p51 heterodimers.[10] Mutation N348I decreases susceptibility to NVP 7.4-fold and to EFV 2.5-fold and enhances NNRTI resistance in the context of K103N.[7] Mutation T369I reduces susceptibility to EFV 3-fold.[11] The double mutant T369I/N348I causes fold change of 11 and 60 to EFV and NVP, respectively. Mutation E399G increases EFV resistance 3.6-fold and reduces viral replication capacity when associated with other mutations in the NNRTI binding pocket.[12]

Despite the knowledge on the effect of RT-C and RT-R mutations on NRTI and NNRTI susceptibility *in vitro* is increasing and becoming more refined, the impact of these mutations on ART outcomes *in vivo* remains largely unknown.

OBJECTIVE

To investigate the association between the RT-C mutations N348I, T369I, E399G/D, G335C/D, A360I/V, V365I, A371V, A376S, and the RT-R mutation Q509L with risk of virological failure to first NNRTI-based ART.

To evaluate the coselection of RT-C and RT-H mutations with IAS-USA mutations in the RT.

METHODS

Subjects: This study included NNRTI-naive patients enrolled in the EuroSIDA cohorts who: (a) started NNRTI-based 3-drug ART after July 1997, (b) had known pre-NNRTI therapy HIV-1 RNA (VL) and CD4+ counts, (c) had at least 2 VL measures after ART initiation and (d) had a plasma sample available within one year before NNRTI treatment initiation.

Population Sequencing: Blinded pre-NNRTI population sequencing of plasma HIV-1 comprising the protease (PR) and RT-coding regions of *pol* was performed using the Trugene™ HIV-1 Sequencing Kit (Siemens Medical Solutions) plus a home-brew population sequencing method that included the C-terminal region of RT, i.e., Connection (RT codon: 289–423) and RNaseH (RT codon: 424–560) domains. Codon changes in the RT C-terminus relative to the corresponding consensus reference sequence were identified automatically with the HIVdb Program, available at the Stanford HIV Drug Resistance Database website (http://hivdb.stanford.edu/pages/algs/HIVdb.html).

Statistical analyses: Standard descriptive statistics were used to illustrate the population characteristics. Virological failure was defined as two consecutive VL determinations above 500 copies/mL after NNRTI initiation (at least 6 months after initiation if the baseline viral load was above 500 copies/ml). Time to virological failure (the first of these two measurements) was evaluated using Cox regression models, stratified by clinical centre and adjusted for previous use of ART and AIDS diagnoses, year of NNRTI initiation, CD4 count at baseline, nadir CD4 count, VL at baseline, maximum VL, number of non-NNRTI active drugs in the regimen and NNRTI predicted susceptibility (both estimated using Rega v7.1), with follow-up time right censored at the time of the penultimate available viral load measurement. A separate model for each of the candidate RT-C and RT-R mutations was fitted with and without testing for the interaction with specific NNRTI used. A Wald test was used to test the significance of the interaction.

Covariation between RT-C / RT-H mutations and IAS-USA resistance mutations in the RT was evaluated using the correlation Phi statistic, which takes value in the range [-1;+1]: phi=-1 means that mutations are negatively associated (i.e. two mutations are antagonists), phi=0 indicates no association whereas phi=+1 means that mutations are positively correlated (i.e. they cluster). The p-values were adjusted with the Benjamini-Hockberg method. The Covarius package in "R" developed by T Sing was employed.

RESULTS

Table 1 displays the characteristics of subjects. Sequence data were obtained for 287 patients; 115 subjects started NVP, and 172 EFV. Sixteen percent of subjects were previously ART-naive (9% NVP, 22% EFV, p<0.001). Subjects starting NVP initiated NNRTI ART significantly earlier than those starting EFV, had greater previous exposure to NRTIs and were more likely to initiate PI therapy concurrently with NNRTIs. Marginal statistical significance was observed toward lower baseline CD4+ counts and lower number of active non-NNRTI drugs included in the new regimen (estimated by Rega v7.1) in subjects in the NVP arm, relative to those initiating EFV.

In 162 (56%) subjects, the M184V mutation was detected (66 (57%) NVP, 96 (56%) EFV) and in 178 (62%) at least one TAM was detected (78 (68%) NVP, 100 (58%) EFV).

Prevalence of connection and RNAseH mutations at the initiation of NNRTI therapy (Figure 1)

Despite the fact that subjects in the NVP arm were having more extensive previous exposure to NRTIs than that in those who started EFV, frequencies of RT-C and RT-H mutations were similar in both NVP and EFV groups (p=0.185). The most frequent RT-C mutations were A371V=23%, G335C/D=14%, and A376S=9%. Regarding IAS-USA RT mutations, 162 subjects (56%) had the M184V mutation (66 (57%) NVP, 96 (56%) EFV) and 178 (62%) had at least one TAM (78 (68%) NVP, 100 (58%) EFV).

Covariation between mutations in the connection and RNAseH domains and RT IAS-USA mutations (Table 2, Figure 2).

Figure 2 shows the results of the covariation analysis between connection and RNAseH mutations with RT IAS-USA mutations using the Phi correlation analysis. As expected, the TAM1, TAM2 and 151M-type clusters can be identified as visible "hot spots". After p-value adjustment, a number of weak correlations were identified between some RT-C and IAS-USA mutations (**Table 2**). Mutation V365I was weakly associated with L210W (phi=0.23, B-H adjusted p=0.02), mutation A371V was associated with M41L (phi=0.21, p=0.02) and mutation E399D was associated with the 151M-cluster (phi=0.32, p=0.04). In this analysis, A376S did not cluster with any of the RT IAS-USA mutations (phi<0.2).

Table 1

Patient characteristics at the start of the NNRTI-containing regimen

		Total		Nevirapine		Efavirenz		*P*
All		287	100.0	115	40.1	172	59.9	-
Date started regimen (*Median, IQR*)		Nov-00	(Feb-99, Feb-03)	Apr-99	(Jun-98, Jun-01)	Oct-01	(Mar-00, Jan-04)	<0.001
Demographics (*n, %*)								
Male		210	73.2	83	72.2	127	73.8	0.755
HIV exposure	Homosexual	138	48.1	55	47.8	83	48.3	0.309
	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		86	30.0	31	27.0	55	32.0	0.363
Subtype	B	248	86.4	100	87.0	148	86.0	0.826
	Non-B	39	13.6	15	13.0	24	14.0	-
(*Median, IQR*)								
Age (yrs)		41	(35, 47)	41	(34, 47)	41	(35, 46)	0.875
CD4 count (/mm³)	Baseline	240	(160, 340)	210	(144, 320)	252	(171, 362)	0.052
	Nadir	125	(44, 209)	98	(35, 210)	140	(48, 208)	0.310
Viral load	Baseline	4.5	(3.8, 5)	4.4	(3.8, 5)	4.5	(3.8, 5)	0.403
(log_{10}copies/ml)	Max ever	5.1	(4.7, 5.6)	5.1	(4.7, 5.5)	5.1	(4.8, 5.6)	0.391
ARV use at start of regimen (*n, %*)								
Antiretroviral naïve		47	16.4	10	8.7	37	21.5	0.004
Any NRTIs previously used		240	83.6	105	91.3	135	78.5	0.004
Any PIs previously used		201	70.0	84	73.0	117	68.0	0.363
Antiretrovirals in regimen (*n, %*)								
Total no. of drugs in regimen								0.471
3-4		239	83.3	98	85.2	141	82.0	-
>=5		48	16.7	17	14.8	31	18.0	-
Estimated using Rega v7.1:								
No. of active non-NNRTI drugs (*Median, IQR*)		1.5	(0.5-2.0)	1.5	(0.5-2.0)	2.0	(1.0-2.0)	0.056
NNRTI baseline resistance (*n, %*)		176	63.1	77	68.1	99	59.6	0.149

Figure 1

Connection and RNAseH mutations detected at start of NNRTI-containing regimen

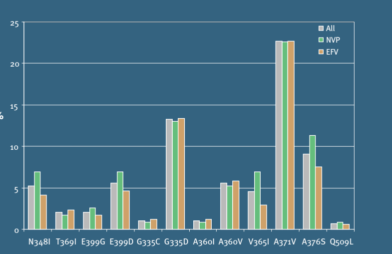

Table 2

Covariation between connection, RNAseH mutations and IAS-USA RT mutations
Table of Phi correlation and adjusted p-values*

Connection and RNAseH mutations	IAS-USA RT mutation	Phi test	B-H adjusted p-value*
335C	219E	0.21	1.00
335D		All <0.2	
348I	67N	0.18	0.13
360I		All <0.2	
360V		All <0.2	
365I	210W	0.23	0.02
369I	188H	0.28	0.76
	190A	0.28	0.76
371V	41L	0.21	0.02
376S		All <0.2	
399D	115F	0.24	0.89
	151M	0.32	0.04
	116Y	0.36	0.02
399G		All <0.2	
509L	103N	0.23	0.96

*The p-values were adjusted with the Benjamini-Hockberg (B-H) method

A total of 231 (80%) had previously started zidovudine or stavudine (ZDV/d4T) before the date of their plasma sample tested for resistance. Of these, V365I and L210W were detected in 10 (4%) patients (p=0.218), A371V and M41L in 41 (18%) (p<0.001), and E399D and 151M in 3 (1%) (p=1.000). No patients in which any of the three clusters were detected were ZDV and d4T naive.

Association between detection of mutations in the connection and RNAseH domains and risk of virological failure (Table 3, Figure 3)

142 (49%) patients experienced virological failure over a median of 19 months of follow-up; virological failure was observed in 77 individuals starting NVP (67%), versus 65 subjects initiating EFV (38%) (p<0.001).

In the multivariable model adjusting for the potentially confounding factors listed in the methods, there was a 2-fold increased risk of virological failure in patients starting NVP versus those starting EFV, RH=2.0 (95% CI: 1.2-3.3, p=0.006). None of the mutations in the connection or RNAseH RT domains evaluated were associated with an increased risk of virological failure independently of the other factors considered (Table 3). After Bonferroni adjustment for multiple comparisons, there was a tendency for mutation A376S to be strongly predictive of virological failure in patients who had started NVP and largely less predictive of failure in those starting EFV. Specifically, detection of mutation A376S was associated with an adjusted RH of virological failure of 10.4 (95% CI: 2.0-54.7, p=0.006) in patients who started NVP and a RH=0.6 (95% CI: 0.1-2.2, p=0.389) in those who started EFV (Bonferroni-adjusted p-value for interaction=0.088).

Similar results were obtained after excluding ART-naive patients, those with IAS NNRTI-associated mutations and those with D67N, K70R or T215F mutations (Figure 3).

In the multivariable model adjusting for potential confounders, all RT-C and RT-R mutations and for the interaction between NVP/EFV use and A376S, the RH of virological failure in those starting NVP versus those starting EFV was 1.7 (95% CI: 1.0-2.8, p=0.039).

CONCLUSIONS

- Mutation A376S in the reverse transcriptase connection domain is associated with more than a 10-fold increased risk of virological failure to NVP-based ART in subjects with prior NRTI exposure; in contrast, A376S does not seem to have a large effect on the virological outcome to EFV-containing ART in our study population. These findings suggest that mutation A376S may reduce HIV-1 susceptibility to NVP. In addition, our results suggest that genotyping the RT connection domain might be useful in subjects with prior NRTI exposure who need to be treated with first-generation NNRTIs
- Given that subjects initiating NVP still had a higher risk of virological failure than those initiating EFV and that the magnitude of the effect was similar to that observed in previous analyses of the database after controlling for the detection of the A376S and other mutations in the connection or RNAseH domains, the effect of such mutations does not seem to fully explain the increased risk of virological failure of NVP regimens relative to EFV in EuroSIDA
- This study did not detect a significant association between mutations N348I, T369I or E399D, shown to decrease NNRTI susceptibility in vitro, and risk of virological failure to NNRTI therapy
- Covariation of RT-C mutations V365I, A371V and G399D with some TAMs confirms previous data suggesting that RT-C mutations are co-selected with TAMs under zidovudine or stavudine exposure

STUDY LIMITATIONS

- The main limitations of our analysis are: (a) the sample size of this study is limited; a larger sample size is needed to decrease the uncertainty around our relative hazards estimates, (b) the NVP and EFV arms are unbalanced regarding several key baseline characteristics; provided that these differences have been properly accounted for by our multivariable analysis, we cannot rule out that there could be other unmeasured confounders (adherence, prescription patterns, etc)
- Currently, there is no in vitro evidence that the A376S mutation is associated with reduced phenotypic susceptibility
- Our findings should be at least replicated in an independent cohort before drawing firm conclusions regarding the clinical utility of routine testing of RT-C and RT-R regions of HIV

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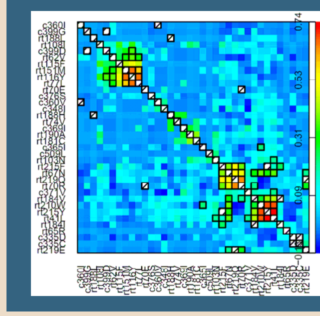
ACKNOWLEDGEMENTS

The EuroSIDA Study Group: **Argentina:** (M Losso), C Elias, Hospital JM Ramos Mejia, Buenos Aires. **Austria:** (N Vetter) Pulmologisches Zentrum der Stadt Wien, Vienna; (R Zangerle) Medical University Innsbruck, Innsbruck. **Belarus:** (I Karpov), A Vassilenko, Belarus State Medical University, Minsk, VM Mitsura, Gomel State Medical University, Gomel; O Suetnov, Regional AIDS Centre, Svetlogorsk. **Belgium:** (N Clumeck) S De Wit, B Poll, Saint-Pierre Hospital, Brussels; R Colebunders, Institute of Tropical Medicine, Antwerp; (L Vandekerckhove) University Ziekenhuis Gent, Gent. **Bosnia:** (V Hadziosmanovic) Klinicki Centar Univerziteta Sarajevo, Sarajevo. **Bulgaria:** K Kostov, Infectious Diseases Hospital, Sofia. **Croatia:** J Begovac, University Hospital of Infectious Diseases, Zagreb. **Czech Republic:** (L Machala) H Rozsypal, Faculty Hospital Bulovka, Prague; D Sedlacek, Charles University Hospital, Plzen. **Denmark:** (J Nielsen) G Kronborg, T Benfield, M Larsen, Hvidovre Hospital, Copenhagen; J Gerstoft, T Katzenstein, A-B E Hansen, P Skinhøj, Rigshospitalet, Copenhagen; C Pedersen, Odense University Hospital, Odense, L Oestergaard, Skejby Hospital, Aarhus. **Estonia:** (K Zilmer) West-Tallinn Central Hospital, Tallinn, Jelena Smidt, Nakkusosakond Siseklinik, Kohtla-Järve. **Finland:** (M Ristola), Helsinki University Central Hospital, Helsinki. **France:** (C Katlama) Hôpital de la Pitié-Salpétière, Paris; J-P Viard, Hôpital Necker-Enfants Malades, Paris; P-M Girard, Hospital Saint-Antoine, Paris; JM Livrozet, Hôpital Edouard Herriot, Lyon; P Vanhems, University Claude Bernard, Lyon; C Pradier, Hôpital de l'Archet, Nice; F Dabis, D Neau, Unité [illegible]

A. Cozzi-Lepri would like to acknowledge Tobias Sing for the use of the Covarius R Programme

Figure 2

Covariation of mutations in the connection and RNAseH domains with IAS-USA RT Mutations



The correlation Phi takes value in the range [-1;+1]; the pair wise Phi test value is shown in colors

phi=-1 means that mutations are negatively associated (i.e. two mutations are antagonists),

phi=0 indicates no association

phi=+1 means that mutations are positively correlated (i.e. they cluster).

Color code: red shades for the highest positive correlations, green for medium positive, blue for low correlations

Table 3

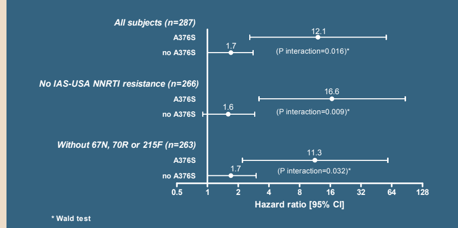
Relative hazards of virological failure after starting a regimen containing any NNRTI, NVP or EFV

Mutation	Any NNRTI n	Univariable RH	Univariable 95% CI	Multivariable* RH	Multivariable* 95% CI	Nevirapine n	Univariable RH	Univariable 95% CI	Multivariable* RH	Multivariable* 95% CI	Efavirenz n	Univariable RH	Univariable 95% CI	Multivariable* RH	Multivariable* 95% CI
N348I	15	1.7	0.8-3.5	1.1	0.5-2.4	8	1.1	0.4-3.1	1.0	0.3-3.4	7	1.7	0.5-5.6	1.3	0.3-5.7
T369I	6	1.4	0.5-3.9	1.4	0.4-5.4	2	1.2	0.2-9.5	8.0	0.8-79.3	4	2.0	0.5-8.4	2.7	0.2-32.6
E399G	6	1.1	0.3-3.9	1.1	0.2-6.2	3	2.5	0.5-13.1	2.1	0.2-28.4	3	-	-	-	-
E399D	16	2.2	1.0-4.6	1.5	0.7-3.4	8	1.9	0.6-5.6	1.9	0.6-6.4	8	2.1	0.7-6.9	1.8	0.5-6.2
G335C	3	1.1	0.2-4.9	1.1	0.2-5.4	1	1.1	0.1-12.7	0.9	0.1-12.2	2	1.7	0.2-12.6	2.1	0.2-19.3
G335D	38	0.9	0.5-1.5	1.1	0.6-2.0	15	1.2	0.6-2.5	0.9	0.4-2.1	23	0.4	0.1-1.0	0.5	0.1-1.7
A360I	3	0.9	0.1-6.5	1.9	0.2-15.1	1	2.0	0.2-16.0	16.0	1.0-270	2	-	-	-	-
A360V	16	0.8	0.4-1.9	1.0	0.4-2.2	6	0.5	0.1-1.7	0.8	0.2-3.8	10	1.0	0.4-3.0	1.2	0.3-4.3
V365I	13	1.8	0.8-4.0	1.9	0.7-5.0	8	2.1	0.6-6.5	0.6	0.1-3.1	5	0.9	0.1-7.0	2.0	0.2-17.8
A371V	65	1.9	1.2-2.8	1.7	1.0-2.7	26	2.9	1.5-5.6	2.8	1.2-6.4	39	1.4	0.8-2.6	1.5	0.7-3.4
A376S	26	1.4	0.7-2.8	1.0	0.5-2.3	13	2.6	0.9-7.2	10.6	1.8-61.4	13	0.7	0.2-2.5	0.6	0.1-2.8
Q509L	2	-	-	-	-	1	-	-	-	-	1	-	-	-	-
G335D*A371V	12	0.6	0.2-2.1	0.5	0.1-2.2										
A376S*A371V	7	0.4	0.1-1.6	0.2	0.0-1.5										
G335D*A376S	6	0.7	0.2-3.5	0.5	0.1-2.9										

*After adjustment for number of previous drugs taken (NRTIs and PIs), previous AIDS diagnoses, year started NNRTI, CD4 count (baseline, nadir), viral load (baseline, max.) and number of other drugs in regimen (NRTIs and PIs, plus number of non-NNRTI active drugs in regimen and NNRTI baseline resistance (both defined by Rega v7.1).

Figure 3

Risk of virological failure in subjects starting nevirapine vs. efavirenz in the presence or absence of the A376S mutation



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