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ORIGINAL INVESTIGATION

The SnoB study: frequency of baseline raltegravir resistance mutations prevalence in different non-B subtypes

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Abstract The SnoB study analysed the variability of the integrase (IN) gene of non-B viruses from treatment-naïve patients to determine whether non-B subtypes carry natural resistance mutations to raltegravir (RAL). Plasma viral RNA from 427 patients was gained, and IN sequences were subtyped and screened for subtype-specific highly-variable residues. Seven viruses of different subtypes were phenotypically tested for RAL susceptibility; 359/427 samples could be sequenced. One hundred and seventy samples (47%) were classified as non-B subtypes. No primary RAL resistance–associated mutations (RRAMs) were detected. Certain secondary mutations were found, mostly related to specific non-B subtypes. L74 M was significantly more prevalent in subtype 02_AG, T97A in A and 06_cpx, V151I in 06_cpx, and G163R in 12_BF. Various additional mutations were also detected and could be associated with the subtype too. While K156 N and S230 N were correlated with B subtype, V72I, L74I, T112I, T125A, V201I and T206S were more frequent in certain non-B subtypes. The resistance factors (RF) of 7 viral strains of different subtypes ranged from 1.0 to 1.9. No primary or secondary but subtype-associated additional RRAMs were present. No correlation between RF and additional RRAMs was found. The prevalence of RRAMs was higher in non-B samples. However, the RFs for the analysed non-B subtypes showed lower values to those reported relevant to clinical failure. As the role of baseline secondary and additional mutations on RAL therapy failure is actually not known, baseline IN screening is necessary.

Keywords Integrase inhibitors · Raltegravir · Polymorphisms · Non-B subtypes · Resistance mutations · Phenotypic analysis

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Introduction

The HIV-1 integrase inhibitor (INI) raltegravir (RAL, MK-0518, Isentress®) is indicated for the treatment of adult HIV-infected patients in combination with other antiretroviral agents. RAL blocks the strand-transfer activity of the viral integrase (IN), necessary for the integration of the proviral DNA into the host genome. In addition, other strand-transfer inhibitors are already in clinical studies [1–7].

So far, clinical studies illustrating the effectiveness of RAL were focused on HIV-1 subtype B [3, 4, 8–11].

However, more than 90% of HIV-1-infected people worldwide harbour non-B subtypes [12]. Caused by migration movements and international tourism, a large number of HIV-positive patients with different non-B subtypes are meanwhile treated in Germany. The non-B prevalence in the German State of NRW (RESINA Study) from 2001 to 2009 was on average 28% (SD 3.9%).

As known for the protease and the reverse transcriptase, subtype-specific mutations in the target gene may also have an impact on the activity of antiretroviral drugs [13, 14].

The purpose of the SnoB study was to analyse the variability of the HIV-1 IN gene of non-B subtypes from antiretroviral treatment-naïve patients to determine whether non-B subtypes carry natural resistance-associated mutations to RAL.

Methods

The SnoB study is a prospective cohort study enrolling HIV-1-infected patients being treated in 24 German centres. Patients included in this study were suspected to carry non-B subtype viruses, therapy-naïve for all classes of antiretroviral drugs and signed an informed consent prior to any study procedures.

At the Institute of Virology of the University of Cologne, 427 plasma samples were analysed centrally. Viral RNA was isolated, amplified and sequenced as described recently [15]. Each IN sequence was subtyped with the geno2pheno$_{[integrase]}$ tool (http://integrase.bioinf.mpi-inf.mpg.de/index.php) and screened for mutations in comparison with the subtype B reference strain HXB2 to identify subtype-specific polymorphisms. It was analysed whether the detected variations corresponded to reported RAL resistance–associated mutations (RRAMs). The detected B sequences served as comparison group.

RRAMs were classified in primary, secondary and additional mutations, according to Sichtig et al. [15]. Briefly, primary RRAMs significantly increase the IC$_{50}$ for RAL and are selected in vivo under RAL treatment. Secondary RRAMs further increase the IC$_{50}$ for RAL and/or restore the viral fitness of viruses carrying primary mutations. Additional mutations have been selected under RAL treatment in vitro, and their importance for RAL resistance in vivo is still not clear.

M7 cells were used for the phenotypic determination of RAL resistance of 12 viral isolates comprising different subtypes. M7 cells contain a luciferase gene under the control of the HIV-1-LTR so that after integration of proviral DNA into the host cell genome, luciferase is produced. For the RAL susceptibility assay, infectious doses inducing approximately 10,000 relative light units within 3 days were used to infect 25,000 M7 cells cultivated in 200 µl RPMI media supplemented with SPG, 10% FCS, and either without or with increasing concentrations of RAL (from 3.2nM to 1 µM). The IC$_{50}$ values were calculated via the luciferase activity. The IC$_{50}$ shown are mean values from three valid runs.

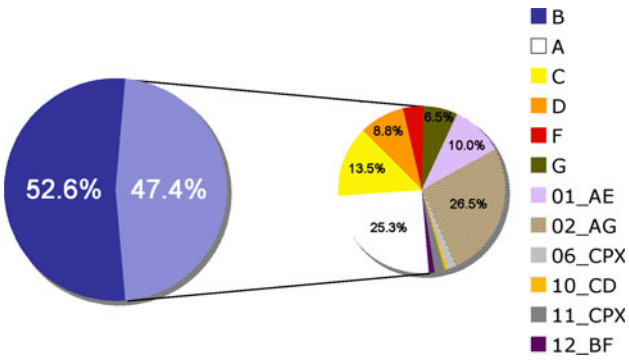

Fig. 1 Prevalence of HIV-1 subtypes in the SnoB Study. SnoB: sequences from 359 therapy naive (naive for all classes) patients attending German HIV centres and included in the SnoB Study

Clinical data like gender, birth date, country of origin, year of first HIV diagnosis, transmission risk, suspected place of infection, CDC stage, CD4$^+$-T-cell counts and viral loads were collected. Clinical data and results of the genotypic and phenotypic tests were compared with each other.

P-values were calculated by Fisher′s exact probability test, 2010 (http://faculty.vassar.edu/lowry/tab2x2.html).

Results

Subtyping

In this study, 427 patients were included; 359/427 samples could be amplified and sequenced; 189 (52.6%) were classified as B; and 170 (47.4%) as non-B subtypes by the geno2pheno$_{[integrase]}$ tool (Fig. 1). The most frequent non-B subtype was the circulating recombinant form (CRF) 02_AG with 26.5% followed by the A subtype with 25.3%. Subtype C was found in 13.5% and the 01_AE in 10% of the analysed non-B samples. The subtypes D and G were also detected in high percentages (D: 8.8%; G: 6.5%). All other subtypes were found in frequencies less than 5%.

Clinical characteristics

The clinical characteristics of the 427 patients are summarised in Table 1.

Although the patients included in our SnoB study were predominantly men (64.2%), the proportion of men and women differed depending on viral subtypes they were carrying; while 88.4% of the patients with B viruses were men, only 51.8% of the patients with non-B viruses were men, so

Table 1 Patients′ characteristics

	All patients	Patients according subtype (n = 359)	
	n = 427	B (n = 189)	Non-B (n = 170)
Gender	26.5% Female	7.4% Female	48.2% Female
	64.2% Male	88.4% Male	41.2% Male
Median (range)			
Age (years)	36 (5 d–77 y)	37 (5 d–77 y)	35 (8 d–71 y)
Time to analysis[a]	4 m (5 d–35 y)	1 m (5 d–19 y)	6 m (8 d–35 y)
VL (copies/ml)	45,900	71,740	48,997
	(<40–6,615,859)	(<40–6,615,859)	(88–163,000)
$CD4^+$-T-cell counts (cells/µl)	330 (3–1,056)	321 (3–989)	309 (8–859)

y years, *m* months, *d* days

[a] time span from first HIV + diagnosis to IN analysis

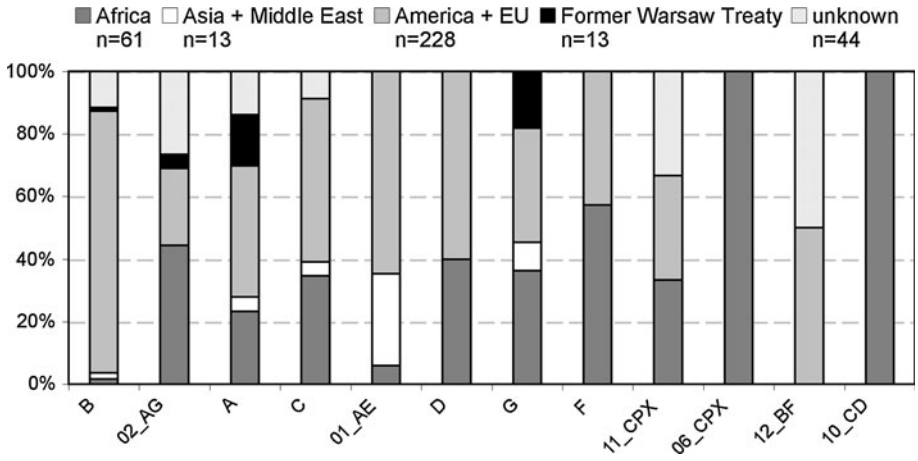

Fig. 2 HIV-1 subtypes: prevalence and origin

the percentage of women infected with non-B subtypes was 6.5-fold higher than B-infected women.

Subtype B-infected patients had in median higher viral loads than patients with non-B subtypes.

Patients harbouring subtype-B strains originated mainly from Europe (83.6%), while those with non-B viruses, comprising many different subtypes, came from all over the world. The CRFs 06_CPX and 10_CD were only detected in patients with an African origin (Fig. 2).

RRAMs

Subtype B viruses displayed a median of 2 (range 0–6) RRAMs per genome while non-Bs showed 3 (0–6). No primary RRAMs were detected (Table 2).

The secondary mutations L74 M, T97A Y143H, V151I and G163R were detected in frequencies ≤2.5% and limited to ≤1 per genome. They statistically correlated with specific non-B subtypes (Table 3). L74 M was present in 8.9% of the CRF 02_AG samples, T97A in 7.0% and 33.3% of the A and 06_cpx isolates (respectively), V151I in 66.7% of the 06_cpx, and G163R in 50% of the 12_BF samples. Y143H was not detected in clade B viruses but in 4.3% of the C viruses, although the different prevalence lacked statistical significance.

Additional RRAMs were also detected, yet in a higher prevalence (≤73.8%) and with a median prevalence of 3 (0–7) mutations/genome in B isolates and 4 (0–6) in non-Bs. Certain additional mutations were associated with specific HIV-1 subtypes. The mutations K156 N and S230 N were present in a significantly higher proportion in the B isolates (Table 2). S119GPRT and V72I were more frequent in subtype B isolates than the total of non-B sequences (Table 2), although they significantly correlated with specific non-B subtypes (Table 3). S119GPRT was found in 51.2% of the subtype A isolates, and V72I was more frequent in subtype C (91.3%). The mutations L74I, T112I, T125A, V201I and T206S were more prevalent in non-B subtypes. The L74I, found in 20% of the non-B isolates (Table 2), had a higher prevalence in subtype A (46.5%) and 02_AG (20.9%). T112I was found in 17.6% of the non-B samples, but in 71.4 and 54.5% of F and G isolates, respectively, and the T206S, detected in only 42.4% of the non-B subtypes, was predominant in the subtypes 02_AG (95.6%), G (90.9%) and F (57.1%). The mutations T125A and V201I, with the highest frequencies in our analysed non-B samples (T125A = 86.5%; V201I = 95.3%), associated with most non-B subtypes. The prevalence of the mutations A128T, M154I, E157Q, V165I, and I203 M could not be significantly associated with any specific subtype.

Table 2 Differences in RRAMs prevalence of B vs. non-B subtypes

	RRAMs*	Subtype		*P* value (two-tailed)
		B (*n* = 189) n (%)	Non-B (*n* = 170) n (%)	
Primary	Y143CR, Q148HKR, N155H	0	0	1.000
Secondary	T66I, E92Q, E138AK, G140AS	0	0	1.000
	L74 M	3 (1.6)	5 (2.9)	0.484
	T97A	2 (1.1)	7 (4.1)	0.091
	Y143H	0	1 (0.6)	0.474
	V151I	4 (2.1)	3 (1.8)	1.000
	G163R	0	1 (0.6)	0.474
Additional	H51Y, T66AK, F121Y, Q146 K, S147G, V249I, R263 K, C280Y	0	0	1.000
	V72I	**132 (69.8)**	91 (53.5)	**0.002**
	L74I	13 (6.9)	**34 (20.0)**	***P* < 0.001**
	T112I	17 (9.0)	**30 (17.6)**	**0.018**
	S119GPRT	**89 (47.1)**	49 (28.8)	***P* < 0.001**
	T125AK	60 (31.7)	**147 (86.5)**	***P* < 0.001**
	A128T	0	2 (1.2)	0.239
	S153AY	0	1 (0.6)	0.474
	M154I	7 (3.7)	3 (1.8)	0.344
	K156 N	**18 (9.5)**	1 (0.6)	***P* < 0.001**
	E157Q	4 (2.1)	5 (2.9)	0.741
	V165I	9 (4.8)	16 (9.4)	0.098
	V201I	104 (55.0)	**162 (95.3)**	***P* < 0.001**
	I203 M	9 (4.8)	8 (4.7)	1.000
	T206S	32 (16.9)	**72 (42.4)**	***P* < 0.001**
	S230RN	**19 (10.1)**	2 (1.2)	***P* < 0.001**

* According to Sichtig et al. 2009

Mutations with statistically significant differences between B and non-B subtypes are highlighted in *bold*

Phenotypic RAL resistance determination

Twelve viral strains corresponding to different subtypes were analysed for phenotypic susceptibility to RAL.

Nine of twelve viral supernatants induced sufficient amounts of luciferase activities after initial infection and could be used for to infect M7 cells in the presence of increasing concentrations of RAL. Six independent runs were performed for each viral supernatant and twelve for the reference virus NL4-3. For the isolate corresponding to the subtype 01_AE, the IC_{50} value could not be calculated due to too low luciferase activities. For all other supernatants, IC_{50} values from at least three independent runs could be calculated. All other runs had to be excluded because the curves cut 50% more than once. The resistance factors (RF) ranged from 1.0 to 1.9 (Fig. 3).

The genotypic analysis of the phenotypic tested samples revealed no primary or secondary but different additional mutations. No correlation between RF and additional RRAMs could be found.

Discussion

The clinical studies conducted to analyse the effectiveness of RAL have been focused on HIV-1 subtype B [3, 4, 8–11]. However, over 90% of HIV-1 infections worldwide and 26.2% of the infected patients in Germany carry non-B viruses.

The SnoB study confirmed the distribution of non-B viruses circulating in therapy-naïve patients in Germany. The high proportion of detected 02_AG isolates in Germany can be related to the high migration rate of patients with an African origin. The total prevalence of B viruses (44.3%) in this study is exceptionally low as predominantly patients with known or assumed non-B infection were enrolled in this study.

Table 3 Prevalence of RRAMs in the specific non-B subtypes versus B isolates

RRAMs		Number of sequences with the specific RRAMs																			
		B (n = 189)	02_AG (n = 45)		A (n = 43)		C (n = 23)		01_AE (n = 17)		D (n = 15)		G (n = 11)		F (n = 7)		06_cpx (n = 3)		11_cpx (n = 3)		12_BF (n = 2)		10_CD (n = 1)	
		n	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value	n	P-value
Secondary	L74 M	3	4	0.027	0	–	1	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–
	T97A	2	2	–	3	0.045	0	–	0	–	0	–	0	–	1	–	1	0.046	0	–	0	–	0	–
	Y143H	0	0	–	0	–	1	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–
	V151I	4	0	–	0	–	0	–	0	–	1	–	0	–	0	–	2	0.002	0	–	0	–	0	–
	G163R	0	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	1	0.010	0	–
Additional	V72I	132	34	–	9	–	21	0.046	4	–	4	–	8	–	6	–	2	–	2	–	0	–	1	–
	L74I	13	9	0.012	20	<0.001	2	–	1	–	0	–	2	–	0	–	0	–	0	–	0	–	0	–
	T112I	17	9	–	2	–	2	–	1	–	4	–	6	<0.001	5	<0.001	1	–	0	–	0	–	0	–
	S119GPRT	89	8	–	22	–	5	–	2	–	4	–	3	–	2	–	0	–	1	–	2	–	0	–
	T125A	60	42	<0.001	38	<0.001	21	<0.001	16	<0.001	1	0.002	7	0.045	3	–	3	0.034	3	0.034	2	–	0	–
	K156 N	18	0	–	1	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–
	V201I	104	45	<0.001	39	<0.001	21	0.001	16	0.001	15	<0.001	11	<0.003	7	<0.002	3	–	1	–	2	–	1	–
	T206S	32	43	<0.001	5	–	3	–	1	–	4	–	10	<0.001	4	0.023	1	–	1	–	0	–	0	–
	S230 N	19	0	–	1	–	0	–	0	–	1	–	0	–	0	–	0	–	0	–	0	–	0	–

P-values values >0.05 (not significant) are indicated as "–"

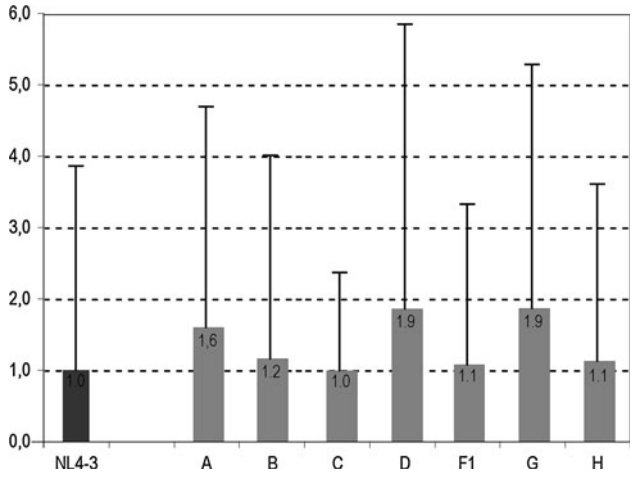

Fig. 3 RAL resistance in cell culture. NL4-3 was used as reference strain ($RF = 1$). IC_{50} values are given as means of all remaining runs, and assay variability is given as standard deviation

Three primary mutations N155H, Q148RHK or Y143RC conferring RAL resistance in vivo have been described [3, 4, 10, 15–17]. In addition, the replacement of one resistance mutational pattern by another has been observed in patients experiencing prolonged virological failure of a RAL-containing regimen [15, 18–20]. Secondary mutations, which further increase the RAL resistance level, are frequently observed in these profiles. E92Q, in addition to being a secondary mutation for RAL *resistance* in vivo *and* in vitro, may play a critical role in the development of resistance to another INI, namely elvitegravir (EVG) [21].

No primary but certain secondary and additional RRAMs were detected in this cohort of therapy-naïve patients, in accordance with previous reports [15, 20, 22–26]. The number and type of RRAMs varied between B and non-B subtypes. B isolates displayed, on average, less RRAMs per genome than non-Bs. No secondary RRAMs correlated with the B subtype, while L74 M, T97A, V151I and G163R could be significantly associated with specific non-B subtypes. For all the subtypes analysed, only the CRF 06_cpx correlated with more than one baseline secondary RRAMs. Concerning the additional mutations, V72I, L74I, T112I, T125A, V201I and T206S could also be related to certain non-B subtypes, and only K156 N and S230RN were significantly more prevalent in B isolates.

In a first attempt to investigate whether these mutational patterns led to different RAL susceptibilities among subtypes, 12 viral isolates corresponding to different HIV-1 clades were tested in a phenotypic resistance assay. The RFs detected in 7 viral isolates ranged from 1.0–1.9, values far below to those reported for the single primary mutations (RF∼13 for N155H and Y143RC and $18 < RF < 38$ for Q148RHK), which correlate with in vivo RAL treatment failure [19, 27, 28]. Taken together, our results support the idea that baseline RRAMs do not seem to severely reduce RAL susceptibility and that RAL may be safely administrated to most patients carrying non-B viruses [3, 4, 29].

However, to date, it is not clear whether baseline RRAMs may have a role in the development of resistance under RAL therapy, as it has been described for PIs, where certain baseline mutations lower the genetic barrier [14]. In fact, secondary and additional mutations do not only affect the RF but also restore or even increase the viral fitness, facilitating the appearance of primary resistance mutations such as the Q148H and the N155H that lead to a 3.4–13.7-fold reduction in the relative viral fitness in comparison with the wild type in the absence of RAL [19, 27, 30]. Indeed, certain studies suggest that baseline RRAMs may have an influence on future RAL resistance development. In a study by Sichtig and colleagues [15], it has been shown that the baseline T206S was not detectable in isolates harbouring the G148HR at RAL failure, but was present in 3/7 samples presenting the N155H mutation. The appearance of the Y143CR at RAL failure correlated with the baseline T97A in 3/4 patients or 1/1 in two previous studies [15, 31]. Ceccerini-Silberstein and colleagues also showed that all three viruses displaying the V165I at baseline developed the N155H at therapy failure. In addition to this, other studies have found RAL therapy failure without the emergence of primary RRAMs [32, 33]. These studies illustrate the potential importance of secondary and additional mutations in contributing to resistance to RAL regimens.

Therefore, although RAL may be safely administrated to most patients carrying non-B viruses, baseline screening should be always performed, in order to identify RRAMs that may influence future RAL resistance development. In addition, prospective studies analysing large numbers of antiretroviral therapy naïve patients taking raltegravir as part of a first-line regimen are mandatory to clarify the clinical significance of baseline RRAMs.

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Ethical standards Ethics approval was given from the responsible ethics committees.

Conflicts of interest The authors have not any commercial or other association that may pose a conflict of interest with this manuscript.

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