

A uniquely prevalent nonnucleoside reverse transcriptase inhibitor resistance mutation in Russian subtype A HIV-1 viruses

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Background: The subtype A variant in the Former Soviet Union (A_{FSU}) causes most of Russia's HIV-1 infections. However, the spectrum of drug-resistance mutations (DRMs) in antiretroviral experienced patients with this variant has not been studied.

Methods: Between 2010 and 2013, genotypic resistance testing was performed on plasma samples from 366 antiretroviral-experienced patients in Siberia.

Results: Three-hundred patients (82%) had subtype A_{FSU} and 55 (15%) had CRF02_AG viruses. The pattern of DRMs was consistent with patient antiretroviral history with one exception. G190S was the most common nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance mutation, occurring in 55 (33%) subtype A_{FSU} viruses from 167 NNRTI-experienced patients compared with none of 37 CRF02_AG viruses from NNRTI-experienced patients ($P < 0.001$). The next most common subtype A_{FSU} NNRTI-resistance mutation, K103N, occurred in 25 (15%) viruses. Wild-type glycine (G) at position 190 is encoded by GGC in more than 99% of published A_{FSU} strains. By contrast, G190 is encoded by GGA or GGG in 97% of other subtypes and in subtype A strains outside of the FSU. Therefore, G190S results from a single G→A transition: G (GGC) → S (AGC) almost exclusively in subtype A_{FSU} viruses.

Conclusion: The predisposition of subtype A_{FSU} to G190S is concerning because G→A is the most common HIV-1 mutation and because G190S causes higher levels of nevirapine and efavirenz resistance than K103N. This study exemplifies the need for characterizing the genetic mechanisms of resistance in diverse populations and warrants studies to verify that NRTI/NNRTI regimens are as efficacious in treating subtype A_{FSU} as viruses belonging to other subtypes.

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Introduction

By 2013, nearly 800 000 persons in Russia were estimated to be living with HIV-1 including about 140 000 persons in the Siberian Federal District [1,2]. By 2012, more than 20 000 persons in this region were receiving antiretroviral therapy [1]. Most HIV-1 strains in Russia and other Former Soviet Union (FSU) countries belong to subtype A1 [3–5]. In the 1990s, this variant (subtype A variant in the Former Soviet Union; A_{FSU}) began spreading widely first in intravenous drug users and then the general population [4].

Additional endemic FSU variants include subtype B, CRF03_AB reported primarily in the western Russian region of Kaliningrad [6], CRF06_cpx reported primarily in Estonia [7], a CRF02_AG variant first reported in Uzbekistan [8] and now widespread in Siberia [9], and CRF63_02A1, a recombinant of this CRF02_AG variant and subtype A_{FSU} [10]. As a result of their relatively recent introduction and spread, several of the HIV-1 strains in Russia and other FSU countries display a strong founder effect. Despite the rapidly expanded use of antiretroviral therapy in the past several years, there are few studies of the genetic mechanisms of resistance in these regions.

Methods

Patients and samples

Patients with virological failure while receiving antiretroviral therapy underwent HIV-1 genotypic resistance testing between 2010 and 2013 at the Siberian Federal District AIDS Centre Laboratory (Omsk, Russia), one of two laboratories in Siberia performing the majority of such testing for routine clinical management. Genotypic resistance testing was performed on 419 samples from 366 antiretroviral-experienced patients. For the 46 patients with more than one sample, only the first was analyzed. Physicians ordering genotypic resistance tests completed a laboratory form indicating the patients' demographics, HIV risk factors, and antiretroviral treatment histories. Patient identifiers were removed prior to analysis. The Siberian Federal District AIDS Centre Laboratory Ethics Committee and the Stanford University Human Subjects Committee approved the study.

HIV-1 genotypic resistance testing

RNA extraction, reverse transcription polymerase chain reaction, protease, and 5' reverse transcriptase (RT) sequencing was performed using either the ViroSeq HIV-1 Genotyping System 2.0 (Celaera Diagnostics, Alameda, California; Abbott Diagnostics, Santa Clara, California, USA) or the AmpliSens HIV-Resist-Seq kit (FBSI, Central Research Institute of Epidemiology, Moscow, Russia). Sequences were analyzed using version 7.0 of the Stanford HIV Drug Resistance Database

(Stanford HIVDB) interpretation program and drug-resistance mutations (DRMs) were defined as any mutation with a penalty score [11]. Sequences were submitted to GenBank under accession numbers KJ870262–KJ870689.

Molecular phylogenetics

HIV-1 subtype was determined using the REGA HIV-1 Subtyping Tool [12]. Unique recombinant forms and those with ambiguous subtypes were examined using the Recombinant Identification Program [13]. Two phylogenetic analyses were performed using the entire protease and first 750 nucleotides of RT.

The first phylogenetic analysis included 300 study sequences and reference sequences belonging to subtypes A, B, C, CRF03_AB, CRF02_AG, and CRF63_02A1. Three viruses with unique recombinant forms and sequences with the highest proportion of ambiguous nucleotides (>1.5% of positions) were not included in the tree. Excluding sequences with the highest proportion of ambiguous nucleotides improved visualization without influencing tree topology. Nucleotides at 21 drug-resistance positions at which five or more patients had a DRM were stripped from the alignment.

The second analysis included global subtype A protease and 5' RT sequences from the Los Alamos National Laboratories HIV Sequence Database (<http://www.hiv.lanl.gov/>; LANL Database). For this analysis, we selected a subset of sequences from this study and subtype A sequences from four regions: Russia, other FSU countries, sub-Saharan Africa, and the remaining regions. To limit the number of sequences, we first excluded sequences having more than one DRM (other than A62V) and having ambiguous nucleotides at more than 1% of positions. Within each region, we then selected representative sequences by randomly excluding individual members of a sequence pair with an uncorrected distance below a prespecified threshold. We used a distance threshold of 3.0% in Russia and other FSU countries, and 6.0% in sub-Saharan Africa and the remaining regions. In addition, subtype A sequences from sub-Saharan Africa were limited to those obtained prior to 2004. The complete set of subtype A LANL sequences and the FASTA file used to create the subtype A tree are in Supplementary Files 1 and 2, <http://links.lww.com/QAD/A584>, <http://links.lww.com/QAD/A585>.

Both phylogenetic analyses were performed with the maximum likelihood program PhyML [14] using the general time reversible nucleotide substitution model and gamma-distributed rate variation among sites. Bootstrap support values were obtained based on the analysis of 100 pseudo-replicates. Trees were drawn with the program FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). The first tree was rooted with African subtype C strain ET2220. The second was rooted with African subtype A2 strain 97DRCTB48.

Statistical analysis

As many patients received more than one antiretroviral regimen, we created a composite treatment history for each patient. We then analyzed associations between RT mutations and previous NRTIs and NNRTIs and between protease mutations and previous protease inhibitors. The frequency of each mutation in subtype A and CRF02_AG sequences was compared with their frequencies in Stanford HIVDB sequences from antiretroviral-naïve and antiretroviral-experienced patients with viruses of the same subtype (<http://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi>) [15]. Comparisons of proportions were performed using the Fisher's exact test as implemented in the software package R [16].

Results

Patients and antiretroviral treatments

Between 2010 and 2013, genotypic resistance testing was performed on samples from 366 antiretroviral-experienced patients. The demographics, HIV risk factors, and antiretroviral treatment characteristics are shown in Table 1. Sixty percent of patients were men and the median age was 30. Nearly one-quarter of patients were infected perinatally. At least 29% were intravenous drug users and at least 18% appeared to be infected heterosexually. HIV transmission risk factors were not reported for 34% of patients.

Fifty-six percent of patients received one antiretroviral regimen, 25% received two regimens, and 19% received three or more regimens (Table 1). The median duration of therapy was 27 months (interquartile range: 11–35). Sixty percent received one or more NNRTIs and 65% received one or more protease inhibitors. Twelve patients received raltegravir and nine received enfuvirtide. The most commonly received NRTIs were lamivudine (3TC), zidovudine (ZDV), didanosine (ddI), and abacavir. Few patients received stavudine (d4T) and none received tenofovir. Efavirenz was received about twice as often as nevirapine. Lopinavir was the most commonly received protease inhibitor.

HIV-1 RT and protease sequences

HIV-1 subtypes included A (300 patients, 82.0%), CRF02_AG (55 patients, 15.0%), B (6 patients, 1.6%), and CRF03_AB (2 patients, 0.6%). Three (0.8%) viruses had unique recombinant forms: A/B in two patients and B/CRF02_AG in one patient. The median uncorrected interpatient sequence distance at unambiguous nucleotide positions was 3.5% for the subtype A sequences and 1.7% for the CRF02_AG sequences.

Figure 1 shows a maximum likelihood tree created with 300 sequences from this study and with reference sequences belonging to subtypes A, B, C, CRF02_AG,

Table 1. Demographics and antiretroviral treatments of 366 patients undergoing HIV-1 genotypic resistance testing, 2010–2013.

		Number	Percentage
Sex	Men	220	60
	Women	146	40
Age	≤15	86	23
	16 to 30	94	26
	31 to 50	176	48
HIV-1 risk factor	≥51	10	3
	Intravenous	101	28
	drug use		
	Mother-to-child transmission	75	20
	Heterosexual	66	18
	Not reported	124	34
Number of regimens per patient	1	206	56
	2	92	25
	≥3	68	19
Cumulative NRTI use	ZDV/3TC	182	50
	ZDV/3TC/ABC	27	7
	ABC/3TC	27	7
	ddl/3TC	24	7
	ZDV/3TC/ddl	21	6
	ZDV/d4T/3TC	18	5
	d4T/3TC	15	4
Other	52	14	
Cumulative NNRTI use	None	148	40
	EFV	137	37
	NVP	62	17
	EFV/NVP	10	3
	Other ^a	9	3
Cumulative protease inhibitor use	None	130	35
	LPV/r	120	33
	ATV	30	8
	FPV	15	4
	LPV/r, ATV	14	4
	LPV/r, DRV/r	11	3
	Other ^b	46	13

3TC, lamivudine; ABC, abacavir; ATV, atazanavir; d4T, stavudine; ddl, didanosine; EFV, efavirenz; FPV, fosamprenavir; LPV/r, ritonavir-boosted lopinavir; NNRTI, nonnucleoside RT inhibitor; NRTI, nucleoside RT inhibitor; NVP, nevirapine; ZDV, zidovudine.

^aIncludes patients who received etravirine usually following EFV or NVP.

^bIncludes patients who received indinavir, nelfinavir, or saquinavir/r usually followed by LPV/r and other protease inhibitor combinations.

CRF03_AB, and CRF63_02A1. Bootstrap values are indicated for branches present in at least 70% of bootstraps. The subtype A viruses share a common ancestor with the subtype A_{FSU} reference strain 10RU6792. The CRF02_AG viruses share a common ancestor with the Russian CRF02_AG and CRF63_02A1 reference strains. Indeed, it may not be possible to distinguish CRF02_AG from CRF63_02A1 strains in Siberia based solely on the protease and 5' RT region [10].

Nonnucleoside reverse transcriptase inhibitor resistance mutations

G190S was the most common NNRTI-resistance mutation, occurring in 55 (33%) subtype A_{FSU} viruses from 167 NNRTI-treated patients (Table 2). G190S was more common in the 167 NNRTI-treated patients with subtype A_{FSU} viruses than the 37 NNRTI-treated

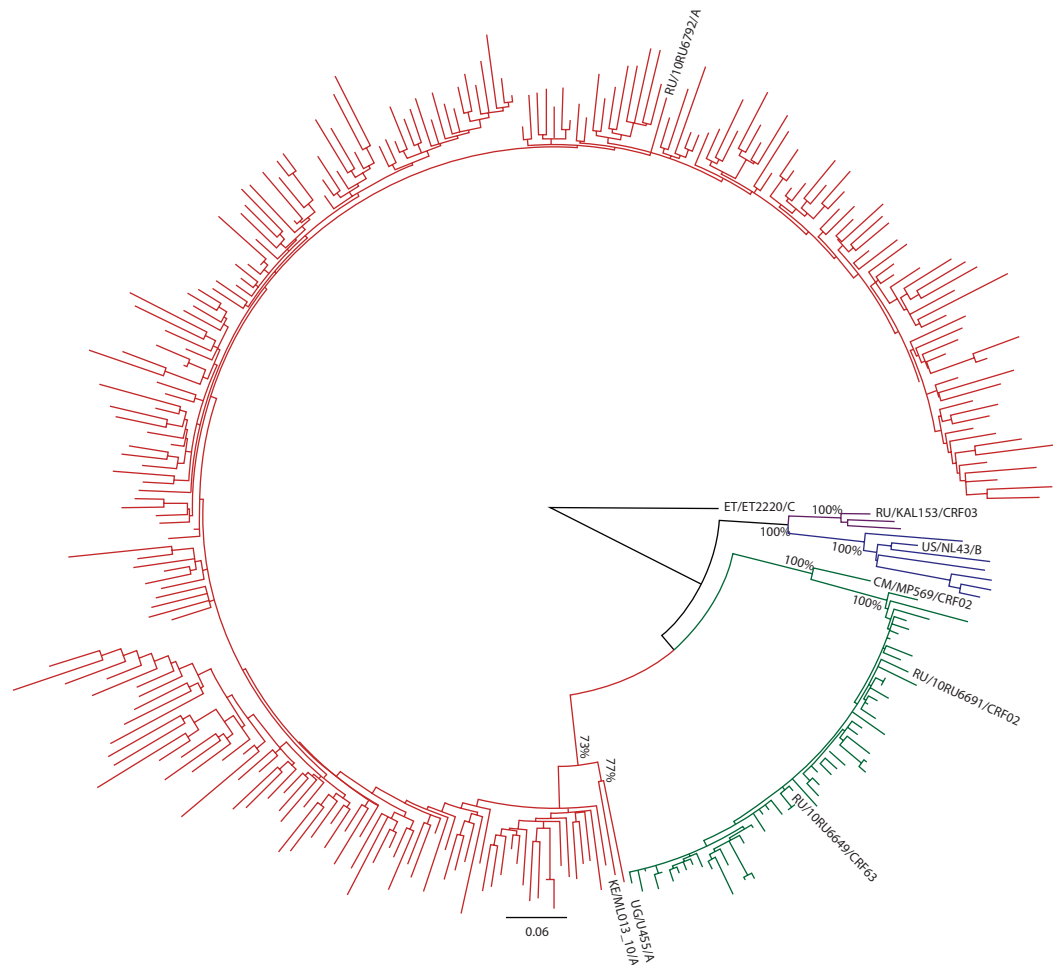


Fig. 1. Maximum-likelihood tree created using 300 sequences from this study and reference sequences belonging to subtypes A (from Uganda and Russia), B, C, CRF02_AG (from Cameroon and Russia), CRF03_AB, and CRF63_02A1. Bootstrap values are indicated for branches present in at least 70% of replicates. Sequences clustering with the subtype A references are in red, with the CRF02_AG and CRF63_02A1 references in green, with the subtype B reference in blue, and with the CRF03_AB reference in purple. The three viruses with unique recombinant forms (two with AB and one with CRF02/B) and the 63 sequences with the highest proportion of mixtures ($\geq 1.5\%$ of nucleotide positions) were not included in the tree. The exclusion of the sequences with the highest proportion of mixtures improved visualization without influencing the tree topology.

Table 2. Most common nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistance mutations in subtype A and CRF02_AG viruses from 204 NNRTI-treated patients.

	Subtype A		CRF02_AG	
	EFV ^a (n = 124)	NVP (n = 43)	EFV ^a (n = 19)	NVP (n = 18)
None	55 (44.4%)	21 (44%)	11 (50%)	3 (20%)
G190S ^b	39 (31.5%)	5 (10%)	0	0
K103N	15 (12.1%)	5 (10%)	5 (23%)	3 (13%)
Y181C	1 (0.8%)	4 (8.3%)	0 (3.8%)	9 (53%)
G190A	0	5 (10%)	0	1 (6.7%)
G190S+K103N	3 (2.4%)	0	0	0
G190S+Y181C	4 (3.2%)	2 (4.2%)	0	0
Other ^c	7 (5.6%)	4 (8.3%)	3 (23%)	2 (6.7%)

EFV, efavirenz; NVP, nevirapine.

^aSixteen of the EFV-treated patients with subtype A viruses and seven of the EFV-treated patients with CRF02_AG viruses also received NVP.

^bTwenty-two of the 39 EFV-treated patients with G190S (without K103N or Y181C) also had K101E. Four of the five NVP-treated patients with G190S (without K103N or Y181C) also had K101E.

^cTwo of the 10 EFV-treated patients with subtype A viruses with other patterns of NNRTI-resistance mutations had G190S.

patients with CRF02_AG viruses (55/167 vs. 0/37; $P < 0.001$). G190S was more common in subtype A_{FSU} viruses from the 124 patients receiving efavirenz ($n = 48$, 39%) than the 43 patients receiving nevirapine ($n = 7$, 16%; $P = 0.008$).

G190S occurred alone in 18 patients and with the accessory resistance mutation K101E in 26 patients. G190S occurred with Y181C, K103N, or another NNRTI-resistance mutation in six, three, and two patients, respectively. K101E was the second most common NNRTI-resistance mutation in subtype A_{FSU} viruses, occurring in 24% ($n = 40$) of viruses from NNRTI-treated patients. The next most common NNRTI-resistance mutations in subtype A_{FSU} viruses were K103N (25 patients), Y181C (13 patients), G190A (8 patients), A98G (4 patients), V108I (3 patients), and Y188L (2 patients). With the exception of G190S and K101E, there were no differences in the proportions of NNRTI-resistance mutations between subtype A_{FSU} and CRF02_AG.

Codon 190 in global HIV-1 sequences

To investigate the high frequency of G190S in subtype A_{FSU} viruses, we examined the codon usage at RT position 190 in HIV-1 sequences in the LANL Database. Among the 4645 full-genome sequences with a wildtype glycine at RT position 190, 88.5% were encoded by GGA, 4.2% by GGC, 6.8% by GGG, and 0.5% GGT. Codon GGC was present in 26% of subtype A sequences, 10% of CRF01_AE sequences, and fewer than 2% of sequences of each of the other subtypes. Viruses with GGC require one nucleotide change at the first position (GGC → AGC) to develop G190S, whereas viruses with GGA require changes at the first and third positions (GGA → AGC).

Among subtype A sequences in the LANL Database encompassing HIV-1 protease and the first 750 RT nucleotides from 3315 individuals, wildtype codon 190 was encoded by GGC in 99.1% (1146/1157) of individuals from Russia and other FSU countries but only 3.2% (71/2157) of individuals from other regions. Figure 2 shows a maximum likelihood tree with 31 representative sequences from this study and 142 representative subtype A sequences from the LANL Database including 48 African sequences, 64 Russian and other FSU sequences, and 30 sequences from Europe, Southeast Asia, and the United States. The subtype A_{FSU} clade had 100% bootstrap support and contained each of the 64 Russian and other FSU strains. Among the 71 non-Russian/FSU sequences for which G190 was encoded by GGC, additional phylogenetic analyses showed that 41 were within the A_{FSU} clade, whereas 30 (including each of the 27 from sub-Saharan African countries) were among the nonclustered basal subtype A sequences.

To determine the contribution of G190S to acquired and transmitted NNRTI resistance, we examined the prevalence of G190S in NNRTI-experienced and NNRTI-naïve patients with subtype A viruses in the Stanford HIVDB. The most common NNRTI-resistance mutations in 725 samples from 647 NNRTI-experienced patients with subtype A viruses were K103N (19%), G190A (13%), Y181C (11%), K101E (4.4%), G190S (3.9%), and Y188L (1.7%) (Supplemental Figure 1, <http://links.lww.com/QAD/A586>). Of 25 patients with G190S, 16 (35%) were from two studies of 46 patients from FSU countries [17,18] compared with 9 (1.5%) of 601 patients from the remaining studies ($P < 0.001$). G190S was not identified in more than 3000 antiretroviral-naïve patients with subtype A viruses including more than 700 patients in FSU countries.

Nucleoside reverse transcriptase inhibitor resistance mutations

M184V/I alone was the most common NRTI-resistance mutation pattern, occurring in 36% (79/218) of patients receiving just thymidine-analog containing regimens (ZDV and/or d4T and 3TC), 23% (14/61) receiving just nonthymidine-analog regimens (abacavir and/or ddI and 3TC), and 14% (12/87) receiving both thymidine-analog and nonthymidine-analog regimens.

One or more thymidine-analog mutations (TAMs) were present in 9% ($n = 20$), 8% ($n = 5$), and 17% ($n = 15$) of patients receiving thymidine-analog, nonthymidine, and both thymidine-analog and nonthymidine-analog regimens. One or more of the non-TAMs, K65R/N, L74V/I, and Y115F were present in 1.4% ($n = 3$), 26% ($n = 16$), and 9.2% ($n = 8$) of patients receiving thymidine-analog, nonthymidine-analog, and both thymidine-analog and nonthymidine-analog regimens. The multi-NRTI resistance mutation Q151M occurred in two patients receiving both thymidine-analog and nonthymidine-analog containing regimens.

A62V, an accessory NRTI-associated mutation endemic in Russian A_{FSU} strains [8,19], was present in 235 (78%) of 300 subtype A viruses and in one (1.8%) of 55 CRF02_AG viruses ($P < 0.001$). There were no other significant differences between subtype A and CRF02_AG NRTI-resistance patterns.

Protease inhibitor-resistance and additional antiretroviral-selected mutations

Of the 243 patients who received one or more protease inhibitors, 32 (13%) had a study-defined protease inhibitor-resistance mutation most commonly L10F [4], K20T [3], V32I [2], L33F [7], M46I/L [9], I47V/A [2], I50L [4], F53L [4], I54V/L [3], Q58E [2], L76V [2], V82A/C [5], I84V [2], L89V [4], and L90M [3].

The nonpolymorphic RT mutation, K82R, occurred in 8.0% (24/300) of subtype A viruses, which is higher than

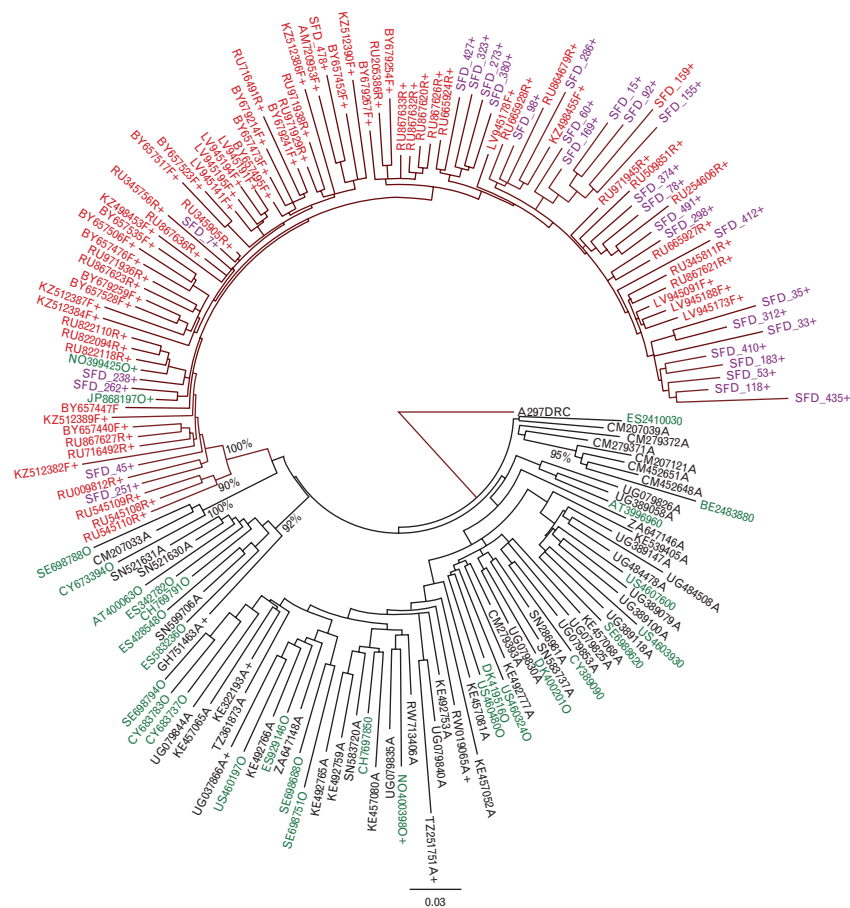


Fig. 2. Maximum likelihood tree of global subtype A sequences. The tree includes 31 representative sequences from this study and 142 representative subtype A sequences from the LANL Database including 48 African sequences, 31 Russian sequences, 33 sequences from other FSU countries, and 30 sequences from Europe, Southeast Asia, and the United States. A subtype A2 sequence (97CDKTB48, GenBank accession AF286238) was used to root the tree. Bootstrap values are indicated for branches present in at least 90% of replicates. The sequences from this study are colored purple and prefaced with SFD (Siberian Federal district). The sequences from Russia and other FSU countries are in red. The sequences from Africa are in black and those from the remaining regions are in green. Each reference sequence is prefaced with the ISO 3166 two-letter country code. Sequences containing the codon 'GGC' at position 190 in RT are followed by a '+' sign.

its proportion in reverse transcriptase inhibitor (RTI)-naïve (10/3072, 0.3%, $P < 0.001$) and RTI-experienced (2/1042, 0.2%, $P < 0.001$) patients with subtype A viruses in the Stanford HIVDB.

Discussion

This study of 366 HIV-1-infected patients with virological failure is the largest study of genotypic resistance in antiretroviral-experienced Russian patients. The fact that G190S was the most common NNRTI-resistance mutation in subtype A_{FSU} strains is notable because globally G190S is not among the most common NNRTI-resistance mutations in NNRTI-experienced patients [20]. G190S also causes higher levels of resistance than most NNRTI-resistance mutations. The median reduction in NNRTI susceptibility by the PhenoSense

assay (Monogram Bioscience, South San Francisco, California, USA) [21] for 14 viruses with G190S and no other major NNRTI-resistance mutation was more than 200-fold for nevirapine and 130-fold for efavirenz compared with 50-fold and 20-fold, respectively, for viruses with K103N alone (<http://hivdb.stanford.edu/pages/phenoSummary/Pheno.NNRTI.Simple.html>). Although G190S does not directly reduce etravirine or rilpivirine susceptibility [22,23], it has a weight of 1.5 in the Tibotec (Janssen Therapeutics, Raritan, New Jersey, USA) etravirine genotypic susceptibility score making it one of the main etravirine-resistance mutations [24].

It has previously been shown that the codon for a wildtype residue predisposes to particular amino acid substitutions at a drug-resistance position. The NNRTI-resistance mutation V106M is more common in subtype C viruses because it requires one nucleotide change compared with two changes in other subtypes [25,26]. By

the same mechanism, the NRTI-resistance mutation V75M is more common in CRF01_AE [27]; and the protease inhibitor-resistance mutation V82M is more common in subtype G [28]. By a different mechanism, subtype C viruses are predisposed to developing the NRTI-resistance mutation K65R [29].

A second difference between subtype A_{FSU} strains and other subtypes is the high proportion of antiretroviral-naïve patients with the NRTI-resistance mutation A62V. Although A62V alone does not reduce NRTI susceptibility, it is an accessory mutation that frequently co-occurs with K65R and Q151M, mutations responsible for multi-NRTI resistance [30,31]. It is not known whether the high prevalence of A62V combined with the predisposition to G190S reduces the genetic barrier to resistance of subtype A_{FSU} strains to NRTI/NNRTI-containing regimens.

G190S was not identified in subtype A viruses in the Stanford HIVDB from more than 700 antiretroviral-naïve patients from Russia and other FSU countries. However, Brenner *et al.* [32] have described a large transmission cluster of viruses in Quebec with G190A, which like G190S is a conservative amino acid substitution suggesting that viruses with G190S may also be transmissible.

Because of the retrospective, laboratory-based nature of this study, we do not have data on the proportion of successfully treated patients in the study catchment area and can make no inferences about the efficacy of first-line therapy. Nonetheless, the predisposition of subtype A_{FSU} to developing G190S demonstrates the need for characterizing genetic mechanisms of resistance in diverse populations of HIV-1-infected patients. The relative ease with which G190S occurs in Russian subtype A strains also suggests the need for retrospective and/or prospective studies to verify that standard NRTI/NNRTI first-line regimens are as efficacious in treating subtype A_{FSU} viruses as they are in treating viruses belonging to other subtypes.

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Conflicts of interest

There are no conflicts of interest.

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