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Minority Human Immunodeficiency Virus Type 1 Variants in Antiretroviral-Naive Persons with Reverse Transcriptase Codon 215 Revertant Mutations▽†

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T215 revertant mutations such as T215C/D/E/S that evolve from the nucleoside reverse transcriptase (RT) inhibitor mutations T215Y/F have been found in about 3% of human immunodeficiency virus type 1 (HIV-1) isolates from newly diagnosed HIV-1-infected persons. We used a newly developed sequencing method—ultradeep pyrosequencing (UDPS; 454 Life Sciences)—to determine the frequency with which T215Y/F or other RT inhibitor resistance mutations could be detected as minority variants in samples from untreated persons that contain T215 revertants ("revertant" samples) compared with samples from untreated persons that lack such revertants ("control" samples). Among the 22 revertant and 29 control samples, UDPS detected a mean of 3.8 and 4.8 additional RT amino acid mutations, respectively. In 6 of 22 (27%) revertant samples and in 4 of 29 control samples (14%; $P = 0.4$), UDPS detected one or more RT inhibitor resistance mutations. T215Y or T215F was not detected in any of the revertant or control samples; however, 4 of 22 revertant samples had one or more T215 revertants that were detected by UDPS but not by direct PCR sequencing. The failure to detect viruses with T215Y/F in the 22 revertant samples in this study may result from the overwhelming replacement of transmitted T215Y variants by the more fit T215 revertants or from the primary transmission of a T215 revertant in a subset of persons with T215 revertants.

Human immunodeficiency virus type 1 (HIV-1) exists in vivo as a quasispecies consisting of innumerable genomic variants evolving through a process of mutation, recombination, and selection. The quasispecies nature of HIV-1 complicates the detection of drug-resistant HIV-1 variants because standard resistance testing using direct PCR dideoxynucleoside chain terminator sequencing rarely detects mutant variants present in fewer than 20% of circulating plasma viruses (14, 20, 26). We previously reported that ultradeep pyrosequencing (UDPS) using the 454 Life Sciences sequencing platform reliably detects minor variants that are missed by direct PCR chain terminator sequencing (26). In this study, we used UDPS to characterize the types of minor variants present in previously untreated HIV-1-infected persons who were found by direct PCR sequencing to have a class of mutations, T215 revertants, that occur almost exclusively among persons primarily infected with a virus previously exposed to selective antiretroviral (ARV) drug pressure.

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MATERIALS AND METHODS

Persons and samples. We studied the treatment histories of all persons receiving medical care at the Kaiser Permanente Medical Care Program of Northern California (KPNC) between 1999 and 2007 who had genotypic resistance testing at Stanford University Hospital (SUH) and who were found to have an amino acid other than T, F, or Y at reverse transcriptase (RT) codon 215 ("215 revertant"). Available cryopreserved plasma samples from persons who were ARV naive, who had no evidence of a mixture that included Y or F, and who had plasma HIV-1 RNA levels ≥4.5 log copies/ml were submitted for UDPS if 40 or more viral templates could be successfully extracted from the cryopreserved sample.

For comparison, samples from 29 ARV-naive KPNC persons lacking T215 revertants or other HIV drug resistance mutations were also submitted for UDPS. These 29 control persons were matched for year of plasma sampling and demographics but had plasma HIV-1 RNA levels that were about 0.5 log copies per ml higher than those with 215 revertants. Moreover, less information on the possible time of infection was known for the control persons than for those harboring T215 revertants. As a laboratory control, a pNL43 HIV-1 clone was sequenced each time UDPS was performed to assess the frequency and distribution of sequence artifact or errors generated by the combined process of nested PCR, emulsion PCR (which is performed immediately prior to pyrosequencing), and the pyrosequencing process itself.

Direct PCR chain terminator sequencing. Genotypic resistance testing for clinical purposes was done on freshly obtained plasma using a previously described standard approach (21). Briefly, RT-PCR products were obtained by plasma virus ultracentrifugation, followed by RNA extraction, reverse transcription using a high-fidelity RT enzyme, and PCR using *Taq* polymerase. Direct PCR bidirectional sequencing encompassing HIV-1 protease and the first 250 to 350 codons of RT was performed using BigDye Terminators with products resolved electrophoretically on an ABI 377 or 3100 sequencer.

UDPS. Fifty-one samples consisting of 22 revertant samples and 29 control samples were submitted for UDPS. Two different laboratory methods were used for UDPS. The first eight samples (which were revertant samples) were sequenced using the 454 Life Sciences (Branford, CT) first-generation GS20 plat-

TABLE 1. PCR amplification strategy for the second set of 43 samples

Target-specific primer[a]	Direction	HXB2 fragment (nt)[b]	Amino acids[c]
CCTCAGATCACTCTTTGGC	Sense	2253→2271	PR1→PR7
ACTYTTGGVCCATCCATTCC	Antisense	2611→2592	RT15→RT21
ATTTTCCCATTAGTCCTATTG	Sense	2545→2565	PR98→RT6
ACTAGGTATGGTAAATGCAG	Antisense	2951→2932	RT128→RT134
CTRGATGTGGGTGATGCA	Sense	2874→2891	RT109→RT114
CNYTATAGGCTGTACTGTCC	Antisense	3284→3265	RT239→RT245

[a] Each primer also comprised one of two adaptor sequences from the 454 Life Sciences platform (GCCTCCCTCGCGCCATCAG and GCCTCCCTCGCGCCA TCAG) and one 4-nucleotide barcode (ACTT, ATCA, TCTG, TACT, CTCT, or CTCA) in the following 5′ to 3′ orientation: 454 adaptor-barcode-primer. Thus, a total of 48 ($3 \times 2 \times 8$) primers were used to encompass protease and 5′ RT and to enable pooling of eight samples.
[b] nt, nucleotide.
[c] PR, protease.

form as previously described (26). Briefly, 1,068-bp nucleotide fragments were generated using nested PCR with the enzyme *Pfu*Ultra (Stratagene). These fragments were nebulized, ligated to adaptors, clonally amplified on capture beads in water-in-oil emulsion microreactors, and pyrosequenced using 1 of 16 lanes of a 40- by 75-mm PicoTiter plate (16).

The subsequent 43 samples were prepared in a different manner and sequenced using a second-generation 454 Life Sciences FLX platform. These samples were amplified using an Expand High-Fidelity PCR System (Roche Diagnostics), which contains a blend of *Taq* and the heat-stable error-correcting polymerase *Pwo*. Although nested PCR was used for all 51 samples, the second round of PCR for the subsequent 43 samples was performed using three sets of HIV-1-specific primers tailed with amplification and sequencing adaptors and 4-bp bar codes from the 454 Life Sciences platform (Table 1). These primer sets ensured complete unidirectional coverage of protease amino acids 8 to 99 and RT amino acids 1 to 238; however, bidirectional coverage was not possible for most of protease and RT.

The decision to switch from the "shotgun" sequencing of nebulized fragments to a targeted amplification approach was made to obtain more even coverage of protease and RT and to enable pooling of samples using the PCR primer bar codes, thus decreasing the need to partition the 454 PicoTiter plates and thereby increasing the number of wells available for sequencing. The decision to switch from *Pfu*Ultra to the Expand *Taq-Pwo* enzyme blend was made to increase the efficiency with which viral templates encompassing protease and RT could be amplified from cDNA despite the known decreased fidelity associated with Expand compared with *Pfu*Ultra (5).

Following RNA extraction and reverse transcription, the cDNA of each of the 51 samples was resuspended in 20 μl of PCR buffer. Four serial dilutions of cDNA were amplified in duplicate using nested PCR with *Pfu*Ultra for the first 8 samples or the Expand PCR system for the subsequent 43 samples to assess the number of distinct viral templates available for sequencing using the 454 system and to determine when it was necessary to use multiple PCRs to obtain a sufficient number of viral templates for sequencing.

Sequencing with the 454 Life Sciences platform generated a standard flowgram file from which nucleotide sequence data encompassing protease positions 8 to 99 and RT positions 1 to 238 and Phred-like quality scores were extracted. Each sequence read was mapped onto the direct PCR sequence using a modification of the Smith-Waterman algorithm that incorporates the Phred-like quality scores into the alignment (26). Sequence reads of lengths less than 80 nucleotides were excluded from analysis because of a reported higher risk of sequence errors associated with shorter reads (10). A median of 630 (interquartile range, 393 to 667) and 1,872 (interquartile range, 1,230 to 3,190) sequence reads per nucleotide was obtained for the first 8 and follow-up 43 samples, respectively.

Statistical analysis. An analysis based on four pNL43 clonal sequences determined using the method applied to the first eight samples yielded an overall mean mismatch error rate of 0.096% (0.07% in nonhomopolymeric regions and 0.4% in homopolymeric regions, where homopolymeric regions were defined as regions with three or more identical consecutive nucleotides and their flanking nucleotides). An identical analysis of the three pNL43 clonal samples sequenced using the method applied to the subsequent 43 sequences yielded a mean mismatch error rate of 0.12% (0.09% in nonhomopolymeric regions and 0.4% in homopolymeric regions). A possible explanation for the similar overall error rates associated with the first 8 and the subsequent 43 samples may be that the high error rate associated with the Expand *Taq-Pwo* blend relative to *Pfu*Ultra (data not shown) is compensated for by a low error rate associated with the newer FLX platform relative to the older GS20 platform.

The distribution of error rates for pNL43 clones sequenced using the method applied to the first eight samples conformed to two Poisson distributions (one for homopolymeric and one for nonhomopolymeric regions). In contrast, the distribution of error rates for the subsequent 43 samples contained several rare outlier nucleotides for which the frequency of differences from pNL43 was slightly higher than would be expected from a Poisson distribution. We hypothesize that these rare errors may have resulted from the increased risk of error during an early round of PCR with the more error-prone Expand *Taq-Pwo* blend.

The statistical approach we used previously to distinguish authentic minor variants (those that were highly unlikely to be explained by Poisson error determined using the pNL43 controls, with a P value of <0.001 without a correction for the 330 amino acids examined) from potential UDPS errors (those that could be explained by Poisson error) (26) was strictly applicable only for the first eight samples. The optimal statistical approach for distinguishing authentic minor variants from potential UDPS errors when an enzyme with lower fidelity than *Pfu* is used requires additional control experiments and statistical modeling (unpublished data). However, for this study, we believe it was reasonable to use a similar cutoff for all 51 sequences for the following reasons: (i) the mean mismatch error rates were similar for the first 8 and subsequent 43 samples (about 0.1%), (ii) the deviation from the Poisson distribution for the subsequent 43 samples was minimal (data not shown), and (iii) there were consistently many more virus templates and many more sequence reads for the subsequent 43 samples than for the first 8 samples, ensuring that a sufficient number of templates were sequenced and improving the robustness of minor variant frequency calculations.

By considering the number of virus templates submitted for UDPS, the distribution of errors on the plasmid clones, and the fact that multiple testing of 330 amino acids was performed, we calculated that variants present at a level of ≥2.0% were highly unlikely to represent sequence artifacts even in homopolymeric regions, making this a conservative cutoff for identifying authentic variants and excluding potential amplification and sequencing errors. Variants present at levels between 1.0% and 1.9% had only a slightly higher theoretical risk of resulting from a sequencing artifact. Therefore, low-prevalence variants occurring at a level of 1.0% or higher at known drug resistance positions (e.g., positions for which there was an a priori suspicion of a possible mutation) were also considered authentic variants.

Confirmatory limiting dilution and molecular clonal sequencing. For four samples with T215 revertants, we performed either limiting dilution clonal sequencing (3 of the first 8 revertant samples) or molecular cloning (1 of the subsequent 14 revertant samples) to confirm the presence of several low-prevalence mutations detected by UDPS but not by direct PCR sequencing. For limiting dilution sequencing, the cDNA product of reverse transcription was serially diluted and subjected to PCR amplification using *Pfu*Ultra with the same primers that yielded the 1,068-bp product. First, eight PCRs were performed per sample at cDNA dilutions of 1:10, 1:30, 1:100, and 1:300. Multiple PCRs were then performed at the dilutions at which fewer than one-half of the reactions were positive. Sequences determined on cDNA dilutions at which fewer than one-third of PCR reactions were positive and which lacked electrophoretic mixtures were considered clonal. Sequences determined on dilutions at which more than one-third of PCR reactions were positive or which contained electrophoretic mixtures were considered to have arisen from two or more cDNA molecules. For the molecular clonal sequencing of one sample, a PCR product encompassing RT positions 1 to 134 was ligated into a TA cloning vector, which was then used to transform competent *Escherichia coli*.

RESULTS

Prevalence of codon 215 revertant mutations. Between 1999 and 2007, plasma samples from 156 (3.4%) of 4,533 KPNC persons undergoing genotypic resistance testing at SUH had one or more sequences with a codon 215 revertant. Seventy-three of these persons were ARV naive at the time their plasma was submitted for sequencing, including 68 whose samples did not also have a Y or F present (in combination with a revertant). The sequences from these 68 persons included 19 with T215D, 15 with T215E, 12 with T215S, 10 with T215C, 4 with T215V, 2 with T215L, 1 with T215N, 1 with T215I, and 4 with mixtures of revertants including SC (2 sequences), DE (1), and DA (1).

Among the 4,533 KPNC persons undergoing genotypic resistance testing between 1999 and 2007, 1,339 persons were ARV naive at the time of their genotyping; 2,120 persons were ARV experienced at the time of their first genotyping; and definitive treatment histories were not available for 1,074 persons although the majority of these persons had some form of poorly documented ARV therapy. Thus, the prevalence of T215 revertants in ARV-naive persons during this 9-year period was 5.1% (68/1,339) among those known to be ARV naive and no lower than 2.8% (68 divided by the sum of 1,330 ARV-naive persons plus the 1,074 persons for whom treatment was not definitively known).

Availability of samples with codon 215 revertant mutations. Plasma HIV-1 RNA levels were ≥4.5 log copies/ml in 46 of the 68 persons with T215 revertants. From these 46 persons, samples with ≥500 μl of cryopreserved plasma were available for UDPS in 25 cases. From these 25 samples, ≥40 viral templates could be extracted and amplified from 22. The median number of extractable and amplifiable virus templates was lower for the first 8 samples for which *Pfu*Ultra was used for amplification (80; range, 40 to 160) than with the subsequent 14 revertant samples for which Expand High-Fidelity PCR was used (160; range, 120 to >480) despite the fact that multiple reverse transcription and PCRs were performed for several viruses in the first set of samples.

Clinical characteristics of persons with codon 215 revertant samples undergoing UDPS. Table 2 shows the year of sampling, the plasma HIV-1 RNA levels, and the CD4 counts for each of the persons with T215 revertants that were available for UDPS. Four persons had been acutely infected within 1 year prior to genotypic resistance testing, three additional persons had been infected some time within the 3 years prior to testing, seven had been infected 2.5 or more years prior to testing, and no information on the timing of infection could be gleaned from the chart review of eight persons.

Direct PCR sequence results of samples with T215 revertants. Each of the 22 sequences belonged to subtype B. Direct PCR sequencing revealed a median of eight differences from the consensus B sequence (range, 2 to 15) including the following 215 revertants: T215C in six persons, T215S in six persons, T215D in four persons, T215E in four persons, T215DE in one person, and T215DA in one person. Seven of 22 samples also had one or more established RT inhibitor resistance mutations by direct PCR sequencing (Table 2) including the nucleoside RT inhibitor (NRTI) mutations M41L (three persons), D67N (one person), L210W (one person), and M41L D67N T69D L210LW (one person) and the non-NRTI (NNRTI) resistance mutation V108I (1 person). In addition, three persons had the nonpolymorphic NRTI-associated mutation H208Y, occurring alone in two persons and with V108I in another person. Protease inhibitor resistance mutations were not identified in any of the 22 T215 revertant samples.

The sequences from samples 7900, 25674, and 27979, which were obtained in 2000, 2005, and 2006, respectively, differed by a mean 1.4% of their nucleotides and formed a clade with 100% bootstrap support in a neighbor-joining tree created from the 22 sequences in this study and 200 randomly selected sequences from untreated persons sequenced at SUH (data not shown). The possibility of an epidemiologic relationship between the persons from whom these samples were obtained was not investigated.

UDPS results of samples with T215 revertants. UDPS identified all 133 RT mutations (6.0 per sample) detected in pure form by direct PCR sequencing and 22 of the 24 mutations detected as part of an electrophoretic mixture. UDPS detected at a prevalence of ≥2.0% a mean of 3.8 RT mutations that were not detected by direct PCR sequencing (range, 0 to 12). UDPS also detected at a prevalence of ≥2.0% the consensus B amino acid at a mean of 1.0 RT positions for which the direct PCR sequence contained a pure mutation (range, 0 to 4) and a mean of 6.8 silent mutations per sample (range, 0 to 35).

UDPS detected additional RT inhibitor resistance mutations in samples from 10 persons including additional T215 revertants in 4 persons, other NRTI resistance mutations in 4 persons, and the minor NNRTI resistance V108I mutation in 2 persons. The additional T215 revertants (Table 2) included T215S in 25% of sequence reads of sample 26420, T215D in 7% of sequence reads of sample 3930, T215C in 1.0% of sequence reads of sample 16412, and T215E and T215S at 1.1% and 1.0%, respectively, of sequence reads of sample 14661. The six additional samples with non-T215-revertant RT mutations included 1816 and 25685, which contained M41L in 11% and 2% of sequence reads, respectively; 23835, which contained M1841 in 1.1% of sequence reads; 30154, which contained K70R, V751, and F77L in 4%, 1.0%, and 1.0%, respectively; and 25674 and 39210, which contained V1081 in 3% and 1.8% of reads, respectively.

T215Y and T215F were not detected at a level above 0.1%, a level far below the reliability cutoff, in any sample. One additional T215 revertant mutation was detected in the sample 14661, T215N at 0.8%. No protease inhibitor resistance mutations were detected at a level ≥1.0% in any of the 22 samples. Of 83 mutations detected only by UDPS, 79 (95%) were present as the dominant variant in ≥0.1% of about 15,000 pooled treated and untreated persons with group M HIV-1 infection in the Stanford HIV Drug Resistance Database (19).

Confirmatory limiting dilution and molecular clonal sequencing. Limiting dilution sequences was determined on the three samples for which additional NRTI mutations were detected by UDPS. For sample 1816 which contained M41L in 11% of pyrosequencing reads, 20 clonal and 4 oligoclonal sequences were performed. M41L was detected in 3 of 20 clones, and M41M/L was detected in one of four oligoclonal sequences. Of the additional RT mutations detected only by UDPS, E36K (2% of pyrosequencing reads), K102R (6%),

TABLE 2. Clinical characteristics and UDPS results for 22 antiretroviral-naive persons with T215 revertants by standard direct PCR Sanger sequencing

Patient no.	Year of sample	No. of mos infected[a]	HIV-1 RNA (log copies/ml)[b]	CD4 count (cells/μl)	Virion count (no.)[c]	T215 revertant[d]	Direct PCR Sanger sequence result[e]	Additional UDPS mutation(s) (% of reads with mutation)[e]
1816	1998	≥36	5.2	360	**160**	DE	83RK	28Q (2), 36K (2), 40A (2), **41L** (11), 42K (2), 102R (6), 123E (6), 142V (12), 173R (15)
3930	1999	<18	5.5	390	**80**	C	83K, 123E, 135T, 177E, 197L, 200TI, 207AE, 211K	20R (21), 215D (7)
5261	1999	NA	4.8	190	**40**	D	20R, 60I, 83K, 123E, 135T, 177E, 202ILF 211K	135V (8)
7900	2000	≥36	5.5	680	**320**	C	68G, 102Q, **108I**, 123E, 135T, 166R, 177E, 178L, **208Y**, 211K	39I (12), 83K (2), 90I (5), 166R (9)
9650	2001	24	4.9	121	**40**	D	**41L**, 60VI, 98S, 135T, 162C, 197E, 211K	189I (3), 200A (8)
14661	2002	NA	>5.7	90	**160**	DA	121Y, 122E, 162C, 177E	172K (6), 178M (3), 200A/I (4/4), 215E/S (1.1, 1.0)
16412	2003	NA	5.4	74	160	S	35M, 39A, **67N**, **70S**, 98S, 102Q, 122E, 123SN, 169D, 177E, 196E, 211RK	166R (3), 200A (5), 215C (1.0)
16407	2003	NA	5.3	133	80	S	20R, 142V, 207E, 211K	None
23835	2004	3	4.3	486	80	S	11R, **41L**, 79ED, 121Y, 122E, 176S, 196E, 211K,	39A (3.2), 162N (2), 177E (2), 178V (5), **184I** (1.1)
18331	2004	NA	>5.7	176	480	S	68G, 122E, 123E, 200A	None
27870	2004	≥30	>5.7	465	160	E	20R, 64KR, 135V, 142M, 162C, 211K	122E (3), K173E (2), 200A (9), 211Q/E/N (10/5/2)
25674	2005	≤36	5.0	310	**40**	C	68G, 102Q, 123E, 135T, 177E, 178L, **H208Y**, 211K	**108I** (3), 200A (5), 189I (2)
25000	2005	3	5.2	170	**160**	E	20R, **41L**, 60I	192N (2)
30215	2005	NA	4.7	228	160	S	60I, 122KE, 135T, 142V, 207E	121H (5)
26420	2005	≥30	4.4	395	160	C	39A, **41L**, **67N, 69D**, 102KR, 104R, 118VI, 135T, 173SN, 200A, 207E, **210LW**, 211KT, 214L	60I (10), 106I (18), 162N (2), 173I (2), 174H (16), 207G/K/D (9/5/5), 210S/R/G (17/3/2), 215S (25)
25685	2005	NA	>5.7	35	480	S	60I, 68SG, 200A, 211K	**41L** (2), 122E (5.0), 211A/Q (10/1.8)
27979	2006	2	>5.7	486	>480	C	68G, 102Q, 123E, 135T, 177E, 178L, **208Y**, 211K	36K (2), 192N (2), 200I (10)
30154	2006	≥3	5.0	76	320	E	16T, 20R, 60I, 83K, 162YH, 211N,	69A (3), **70R** (4), **75I** (1.0), **77L** (1.0), 79D (8), V118I (3), 135T (35)
30195	2006	54	5.3	390	320	E	41L, 50V, 60I, 64R, 104KR, 106VI, 142V, 162C, 211K	11R (3), 48T (2), 139A (2)
37991	2006	6	4.4	673	80	D	68SN, 98S, 122E, 123E, 135T, 207E	126R (3), 146H (2), 177A (5), 207K (10)
39210	2007	NA	>5.7	30	>480	D	6D, 68G, 83KR, 135A, 169DE, 202V, **210W**	60I (10), **108I** (1.8), 122E (6), 135V (10), 200A (9)
42119	2007	≥60	5.2	363	80	C	6D, 8I, 68G, 118I, 135T, 174H, 200TA, 202V, 207E	207G (3)

[a] Estimate of how many months a person had been infected with HIV-1 based on available serological test results. NA, not available.
[b] Plasma HIV-1 RNA level.
[c] Estimated number of independent virus templates submitted for UDPS based on limiting dilution titration. The eight counts in boldface type are from the samples for which cDNA was amplified using *Pfu*Ultr.
[d] Revertant detected by direct PCR sequencing.
[e] Mutations in bold are nonpolymorphic known RT inhibitor resistance mutations. Mutations in underlined and in bold are nonpolymorphic RT inhibitor resistance mutations detected only by UDPS. Underlined but unbolded mutations are T215 revertants detected only by UDPS. The prevalence of minor variants is rounded to the nearest integer except when the prevalence is between 1.0% to 1.9%.

D123E (6%), I142V (12%), and K173R (15%) were detected in one or more limiting dilution sequences whereas E28Q (2%), E40A (2%), and E42K (2%) were not detected in 20 clonal and 4 oligoclonal sequences.

For sample 3930 which contained T215D in 7% of pyrosequencing reads, 45 clonal and 13 oligoclonal sequences were performed. T215D was detected in 2 of 45 clones, and T215D/C was detected in 2 of 13 oligoclonal sequences. Of the additional RT mutations detected only by UDPS, K20R (21% of pyrosequencing reads) was detected in nine clonal and four oligoclonal sequences.

For sample 14661, which contained T215S (1.0%), T215E (1.1%), and T215N (0.8%), each present in about 1% of pyrosequencing reads, 43 clonal and 8 oligoclonal sequences were performed. None of these mutations was detected in the 43 clonal sequences; T215E was detected in two of eight oligoclonal sequences. Of the additional RT mutations detected only by UDPS, I178M (3%), T200A (4%), R172K (6%), and T200I (4%) were detected by limiting dilution sequencing.

For sample 30154 which contained K70R, V75I, and F77L in 4%, 1%, and 1% of pyrosequencing reads, 146 molecular clones, encompassing RT positions 1 to 134, confirmed the presence of K70R in eight clones and V75I and F77L each in one (but not the same) clone. Of the additional RT mutations between positions 1 to 134, T69A (3%), E79D (8%), and V118I (3%) were present in 1, 15, and 3 clones, respectively.

Direct PCR and UDPS sequence results obtained on the 29 control samples. UDPS detected all 116 RT mutations detected in pure form by direct PCR sequencing of the 29 control samples (3.9 per sample) and 37 of 41 mutations detected as part of an electrophoretic mixture. UDPS detected at a prevalence of ≥2.0% a mean of 4.8 mutations that were not detected by direct PCR sequencing (range, 0 to 11) (see Table S1 in the supplemental material). UDPS also detected at a prevalence of ≥2.0% the consensus B amino acid at a mean of 1.1 RT positions at which the direct PCR sequence contained a pure mutation (range, 0 to 5) and a mean of 6.4 silent mutations (range, 0 to 54).

In four control samples, UDPS detected additional RT inhibitor resistance mutations including D67N in 5% of reads in sample 16356; D67N, V108I, and H208Y at 3%, 1.1%, and 1.2%, respectively, of reads in sample 9918; K65R at 1.7% in sample 9635; and the NNRTI-resistance mutation G190E in 1.5% of sample 14177. No protease inhibitor resistance mutations were detected at a level above 1.0% in any control sample. T215Y and T215F were not detected at a level above 0.1%, a level far below the reliability cutoff, in any sample. T215S was detected at a level of 0.8% in one sample. Of 138 mutations detected only by UDPS, 128 (93%) were present as the dominant variant in ≥0.1% of about 15,000 pooled treated and untreated persons with group M HIV-1 infection in the Stanford HIV Drug Resistance Database (19).

The prevalence of minority RT inhibitor resistance mutations at additional RT positions was not significantly higher among the 22 revertant samples (6/22, or 27%) compared with the 29 control samples (4/29, or 14%; $P = 0.4$). However, if the additional T215 revertants (found in four revertant samples) were considered RT inhibitor resistance mutations, then the difference between the two populations would be significantly different (10/22, or 45%, versus 4/29, or 14%; $P = 0.03$).

Response to ARV therapy. Table 3 summarizes the treatments and virological responses of the 22 persons with T215 revertants. Fifteen of the persons received one or more ARV regimens within the year following their genotyping. Ten treated persons sustained complete virological suppression, defined as multiple persistent plasma HIV-1 RNA levels below 75 copies/ml, the lower limit of detection of the bDNA assay (Versant HIV-1 RNA 3.0 assay; Siemens, Berkeley, CA). Two treated persons had initial virological responses followed by planned treatment interruption and virological rebound. Three persons experienced virological failure with the development of resistance. In sample 9650, virological failure occurred after 44 months of virological suppression. However, in samples 16412 and 30154, virological failure occurred upon initial therapy.

Person 16412, whose sequence contained T215S, the NRTI resistance mutation D67N, and the unusual mutation K70S, developed virological failure after treatment with zidovudine plus lamivudine plus nelfinavir. A phenotypic assay following virological failure showed reduced susceptibility to lamivudine and nelfinavir. However, subsequent therapy with tenofovir plus didanosine plus lopinavir-ritonavir led to complete virologic suppression which has persisted for 48 months. Person 30154, whose sequence had T215E and three clonally confirmed minor variants (K70R, V75I, and F77L), developed successive virological failures with tenofovir plus emtricitabine plus efavirenz followed by tenofovir plus emtricitabine plus atazanavir-ritonavir. Genotyping following the second virological failure demonstrated the NNRTI resistance mutations K103N and P225H and the NRTI resistance mutations M184I. V75L but none of the original minority variants (K70R, V75I, and F77L) was detected in this follow-up sample.

DISCUSSION

Biological and clinical significance of T215 revertant mutations. The RT mutations T215Y/F were among the first mutations shown to cause HIV-1 drug resistance (13). Several years after their identification, two laboratories reported that persons infected with viruses containing T215Y but who remained ARV naive evolved unusual mutations at position 215 that were not commonly observed during ARV treatment (7, 9, 29). These mutations, most commonly T215C/D/E/S, were imputed to revert from T215Y to increase virus fitness in the absence of selective drug pressure (7). T215C/D/E/S have been shown to have no effect on HIV-1 drug susceptibility in vitro (8). Nonetheless, T215C/D/E/S have been weakly associated with an increased risk of virologic failure possibly because they are surrogates for the presence of T215Y or because they lower the genetic barrier to the development of T215Y from two nucleotide changes to one nucleotide change (8, 11, 24, 25).

In recent epidemiologic studies, T215 revertants have been found in about 3% of HIV-1 isolates from newly diagnosed HIV-1-infected persons (3, 8, 15, 17, 25, 27, 28). However, there are no published recommendations on how to initiate ARV therapy in persons found to have viruses with these mutations. In this study, we used a newly developed sequencing method to determine whether T215Y could be detected at low levels in persons who have a T215 revertant detected by direct PCR sequencing. The finding of T215Y/F or other drug resistance mutations in persons with T215 revertants would suggest

TABLE 3. ARV therapy and virologic response in 22 persons with T215 revertant mutations undergoing UDPS

Patient no., treatment, and response group	Year of sample	CD4 count (cells/μl)	HIV-1 RNA (log copies/ml)[a]	Drug resistance mutation(s) (including minority variants)[b]	ARV[c]	Description of virological response and follow-up therapy[c,d]
Treated, complete virological response (n = 10)						
1816	1998	360	5.2	41L (11%), 215D	d4T + ddI + EFV	Complete virologic suppression at 54 mo
3930	1999	390	5.5	215C, 215D (7%)	d4T + 3TC + IDV	Complete virologic suppression at 18 mo
5261	1999	190	4.8	215D	d4T + 3TC + EFV	Complete virologic suppression at 15 mo
14661	2002	90	>5.7	215DA, 215S/E (1%, 1%)	ZDV + 3TC + LPV/r	Complete virologic suppression at 21 mo
16407	2003	133	5.3	215S	TDF + 3TC + EFV	Complete virologic suppression at 34 mo
18331	2004	176	>5.7	215S	ddI + 3TC + EFV	Complete virologic suppression at 42 mo
25000	2005	170	5.2	41L, 215E	ZDV + 3TC+ ABC + LPV/r	Complete virologic suppression at 22 mo
25685	2005	35	>5.7	41L (2%), 215S	TDF + FTC + ATV/r	Complete virologic suppression at 31 mo
30195	2006	390	5.3	41L, 215E	TDF + FTC + ddI + EFV	Complete virologic suppression at 14 mo
7900	2000	680	5.5	108I, 208Y, 215C	d4T + 3TC + IDV/r	Complete virologic suppression at 4 mo; ARVs discontinued
Treated, virological failure (n = 3)						
9650	2001	121	4.9	41L, 215D	d4T + ddI +APV	Complete virologic suppression at 44 mo; at mo 8 changed to d4T + ddI + EFV; at mo 44, virologic failure with K103N
16412	2003	74	5.4	67N, 70S, 215S, 215C (1%)	ZDV + 3TC + NFV	Virologic failure with phenotypic 3TC and NFV resistance; follow-up ARV of TDF + DDI + LPV/r, leading to virologic suppression at 60 mo
30154	2006	76	5.0	70R (4%), 75I (1%), 77L (1%), 215E	TDF + FTC + EFV	Virologic failure on initial and follow-up with TDF + FTC + ATV/r; repeat genotype after second virologic failure detected V75L K103N M184I P225H
Untreated or early discontinuation (n = 9)						
27870	2004	465	>5.7	215E	ABC + 3TC + LPV/r	Partial virological response; ARVs discontinued after 2 mo
39210	2007	30	>5.7	108I (2%), 210W, 215D	TDF + FTC + LPV/r	Lost to follow-up
23835	2004	486	4.3	41L, 184I (1%), 215S	None	Followed 39 mo; stable RNA and CD4 values
25674	2005	310	5.0	108I (3%), 208Y, 215C	None	Followed 26 mo; stable RNA and CD4 values
26420	2005	395	4.4	41L. 67N, 69D, 210LW, 215C, 215S (25%)	None	Followed 19 mo; stable RNA and CD4 values
30215	2006	448	4.7	215S	None	Followed 15 mo; stable RNA and CD4 values
27979	2006	486	>5.7	208Y, 215C	None	Followed 10 mo; decreasing RNA and stable CD4 values
37991	2006	673	4.4	215D	None	Followed 10 mo; stable CD4 and RNA values
42119	2007	363	5.2	215E	None	Followed 6 mo; stable CD4 and RNA values

[a] Plasma HIV-1 RNA level.
[b] The percentages for the minority variants are shown in parentheses.
[c] 3TC, lamivudine; ABC, abacavir; APV, amprenavir; ATV, atazanavir; d4T, stavudine; ddI, didanosine; EFV, efavirenz; FTC, emtricitabine; IDV, indinavir; LPV, lopinavir; TDF, tenofovir; ZDV, zidovudine; /r, ritonavir provided for pharmacologic boosting. Combined therapies are indicated with a plus sign.
[d] Virologic suppression was defined as persistent plasma HIV-1 RNA levels of <75 copies/ml, the lower limit of quantification of the Siemens bDNA assay.

that persons with T215 revertants may be at increased risk of virologic failure with many standard first-line treatment regimens.

Prevalence of T215 revertant mutations in our population. Our study provides one of the most comprehensive analyses of the prevalence of T215 revertants in a large population of previously untreated individuals in a region in which transmitted resistance is common. The proportion of ARV-naive persons with T215 revertants was estimated to be between 3% and 5%, slightly higher than that reported by other studies. Of the 68 ARV-naive persons with T215 revertants, 59 (87%) had one of the four mutations C, D, E, or S exclusively, whereas 9 (13%) had one of the five mutations I, V, A, L, or N. The nucleotide triplets coding for C, D, E, and N are more similar to those coding for Y than for F; the nucleotide triplets coding for I, V, or L are more similar to those coding for F than for Y, whereas the triplets coding for S and A each have one and two nucleotide differences, respectively, from Y and F.

Minority variants in revertant and control samples from ARV-naive persons. This study is the first study to investigate minority variants in persons with T215 revertants. An unexpected result was that neither T215Y nor T215F was identified as a minority variant in a single person. Moreover, although the samples contain a statistically significant greater number of additional RT inhibitor resistance mutations (45% versus 14%; P = 0.03) than a control population, four of the persons with

additional mutations had additional T215 revertants that may not be clinically significant in the context of a dominant T215 revertant. When persons with additional T215 revertants were excluded from the comparison, there was no difference in the proportion of persons with additional minority variant RT inhibitor resistance mutations compared with untreated control persons (27% versus 14%; $P = 0.4$).

The failure to detect viruses with T215Y in the persons in this study has two possible explanations. First, one or more persons may have been infected primarily with a T215 revertant rather than with T215Y. T215 revertants rarely arise in ARV-treated persons who develop T215Y/F and then discontinue therapy (19). Such a person can transmit a virus containing a T215 revertant to a new person. Second, due to the increased relative fitness of viruses with revertants relative to viruses with T215Y, viruses with a T215 revertant would be expected to eventually outnumber viruses with the transmitted T215Y variant.

In a simplified model of virus competition, an estimated increase in fitness of 5% (which has been reported for T215C/D relative to T215Y [7]) would lower the prevalence of T215Y to below 1% on average after about 90 virus generations or about 4 to 5 months (6). This model, however, is an oversimplification for two reasons: (i) it does not take into account the likelihood that the initially transmitted variant may have a competitive advantage over subsequently arising mutations because of viral interference at the cellular level; (ii) the initially transmitted variant may have a head start at adopting to host immune pressure, which could theoretically counterbalance the decreased fitness of certain transmitted variants. Conversely, this simple model does not take into account the possibility that some infections are oligoclonal and may have already contained a T215 revertant or that the estimates of the decreased fitness of T215Y/F relative to the T215 revertants may be even lower than the estimates based on in vitro studies.

The failure to detect T215Y or F cannot be explained by a lack of sensitivity with our experimental approach. For nearly all samples, several lines of evidence suggest that our experimental approach would reliably detect low-prevalence variants present at levels in the range of 2% or lower. First, our limiting dilution analysis performed to quantify the number of variants submitted for UDPS showed that, with the exception of three of the first eight samples which had low cDNA viral numbers (approximately 40 viral copies), the remaining samples had cDNA numbers of ≥80 viral copies. Second, UDPS reliably detected low-prevalence amino acid variants at levels between 2% to 5% in 18 of 22 samples and at levels between 2% to 10% in 20 of 22 samples. Although low-prevalence amino acid variants were not detected in two samples (samples 16407 and 18331), sample 16407 contained 11 low-prevalence synonymous nucleotide variants with prevalences ranging from 2% to 10%. Finally, T215Y or T215F was not detected in the four samples for which multiple clones were sequenced.

A recently published paper also suggests that our findings may not be as unexpected as we first believed. A recent summary of the RT mutational patterns in 46 primarily infected French persons with RT inhibitor resistance indicated that, whereas 15 persons contained T215Y or F in their direct PCR sequence, an even larger number, 19, contained only a revertant mutation at this position (4). The high prevalence of revertants in the recently infected persons in this study raises the possibility that viruses with revertant mutations may be more likely to be transmitted than T215Y or F (as revertant mutations are far less common than Y or F among ARV-treated persons [http://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi]) (19) or that T215Y and F may mutate rapidly to one of the revertants following transmission.

Sensitivity and specificity of UDPS for detecting minority HIV-1 variants. The sensitivity of plasma HIV-1 UDPS is limited primarily by the number of cDNA templates that can be extracted and amplified from a plasma sample. Despite the high HIV-1 RNA levels (≥4.5 log copies/ml) in the plasma samples in this study, the approximate number of cDNA templates submitted for pyrosequencing ranged from 40 to >480 copies. Factors contributing to the relatively low yield of cDNA template relative to the plasma HIV-1 RNA level include the low efficiency (~2%) of RNA extraction and reverse transcription of HIV-1 protease and RT nucleic acid from cryopreserved plasma samples (22) and the low volumes of plasma, extracted RNA, and cDNA for which most PCR and sequencing protocols have been optimized.

The PCR amplification step also plays an important role in determining how many viral templates can be submitted for UDPS. Indeed, it was only possible to consistently recover a sufficiently high number of templates from the majority of stored samples after we changed our PCR amplification enzyme from *Pfu*Ultra to the Expand High-Fidelity *Taq-Pwo* blend. Although the *Taq-Pwo* blend has a higher error rate than *Pfu*Ultra (5 and data not shown), its processivity is higher presumably because the 3′ to 5′ exonuclease activity of *Pfu* often degrades oligonucleotides that prime DNA synthesis, particularly when there are mismatches between the PCR primer and template (23).

The specificity of UDPS is limited by the errors occurring during PCR amplification (2) and during pyrosequencing (10, 16, 26). Specificity can be improved by using high-fidelity enzymes such as *Pfu* (at the cost of poorer template recovery), using bidirectional sequencing, employing heuristic approaches to detect canonical 454 sequencing errors such as carry-forward and incomplete extension errors that are commonly found in homopolymeric regions (10), increasing sequence coverage, and raising the sensitivity threshold so that only mutations occurring above a threshold proportion of reads are considered authentic (26).

Sensitivity and specificity can differ by severalfold across a sequence and across different samples because sequence errors are more common in homopolymeric regions and because it is usually not possible to create precisely equimolar concentrations of DNA for each of the amplicons submitted for UDPS. Because there are many factors complicating the determination of sensitivity and specificity, additional studies and additional statistical modeling will be required before the ideal cutoffs for distinguishing authentic variants from sequence error will be developed. Therefore, for this study, we have chosen the conservative cutoffs of 1.0% at drug resistance positions and 2.0% at all other RT positions, while still reporting all codon 215 mutations including those occurring below a level of 1.0%. The high levels of agreement between the UDPS results and the confirmatory clonal sequencing performed on four samples support the cutoffs we have chosen.

Insight into the population genetics of low-prevalence HIV-1 variants. Although we were not able to detect variants present at levels below 1% to 2%, our study provides several novel insights into the population genetics of minority HIV-1 variants in untreated individuals particularly at RT position 215. First, nearly all variants present at low levels within individuals are variants that have been reported as the dominant variant within other individuals. Specifically, 94% of the variants in the 51 untreated persons in this study occurred in pure form (not as an electrophoretic mixture) in ≥0.1% of pooled treated and untreated group M sequences from 15,000 HIV-1-infected individuals. Second, T215Y/F, the T215 revertants, and probably most drug resistance mutations do not appear to occur as naturally occurring low-prevalence variants.

Among the 51 previously untreated persons in this study, UDPS detected about seven additional minority amino acid variants per sequence (including protease and RT) compared with direct PCR sequencing. In contrast, we recently described the detection of 18 additional minority amino acid variants per sequence in a study of heavily ARV-experienced HIV-1-infected persons (26). The increased number of minor variants in heavily treated persons is likely to result in part from known drug resistance mutations and accessory drug resistance mutations as well as hitchhiker mutations resulting from serial bottlenecks occurring during therapy. Moreover, among the 22 persons with T215 revertants, 7 were known to have been infected within the previous 3 years—another factor that likely to influence viral diversity and the proportion of positions with low-prevalence mutations.

Virological response to therapy in persons with T215 revertant mutations. The effect of T215 revertants on the virologic response to a new ARV treatment regimen has been less marked than that of other drug resistance-associated mutations. Whereas transmitted NNRTI-associated mutations such as K103N have been shown to be strong predictors of virologic failure in persons receiving an NNRTI-containing regimen (1, 12), transmitted T215 revertants have had a less pronounced effect (1, 4, 17, 18, 24, 25). In our study, the overwhelming majority of persons with T215 revertants who received therapy experienced sustained virologic suppression. Only two persons developed rapid virological failure with drug resistance that appeared possibly to be related to the presence of low-prevalence mutations that accompanied the T215 revertant, although one of these persons received a regimen that is currently considered suboptimal (zidovudine plus lamivudine plus nelfinavir) and the other had been nonadherent with therapy. Moreover, in several persons, knowledge of the T215 revertant and in some cases of accompanying additional drug resistance mutations may have prompted the use of a ritonavir-boosted protease inhibitor (used in six persons) or an NRTI backbone consisting of three rather than two drugs (used in two persons).

In conclusion, our UDPS results suggest that the presence of T215 revertants in previously untreated persons is usually not associated with T215Y/F or a marked excess of additional drug resistance mutations present as minority variants. The generally successful treatment of persons with these mutations is consistent with our laboratory studies. However, the few cases in which multiple additional mutations were detected and the two cases in which virological failure occurred rapidly suggest that initial treatment of persons with T215 revertants should probably consist of a regimen with a higher than usual genetic barrier to resistance such as two NRTIs plus a boosted protease inhibitor or perhaps three NRTIs plus an NNRTI. Considering the large numbers of previously untreated persons with these mutations, additional retrospective studies or even a prospective study of initial treatment in persons with T215 revertants would be clinically useful.

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