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# Substitutions in the Reverse Transcriptase and Protease Genes of HIV-1 Subtype B in Untreated Individuals and Patients Treated With Antiretroviral Drugs

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## Abstract

The nucleotide transition G→A is known as a hypermutation due to its high prevalence in HIV-1 and other pathogens. However, the contribution of the G→A transition in the generation of drug resistance mutations is unknown. Our objective was to ascertain the rate of nucleotide substitutions in protease (PR) and reverse transcriptase (RT) in both untreated and treated HIV-1 patients. Genotypic analysis was performed on viruses from both treated and untreated patients with subtype B infections. Nucleotide genomic diversity was compared with a consensus subtype B reference virus. Then, the prevalence of resistance-associated mutations in different subgroups of treated patients was evaluated in relation to the patterns of nucleotide transitions. In untreated patients (n = 50) G→A was most prevalent, followed by A→G, C→T, and T→C transitions. In treated patients (n = 51), the prevalence of A→G was similar to that of G→A. Among mutations that confer resistance to antiretroviral drugs, M184V was present in 76% of treated patients and K70R in 31% (A→G transitions). Other frequent mutations in RT included T215Y (C→A and A→T substitutions), which was prevalent in 31% of treated patients. In PR, a L90M (T→A substitution) was prevalent in 47% of protease inhibitor (PI)-treated patients. In conclusion, the G→A transition was most prevalent in RT and PR among untreated patients. In contrast, A→G was the most prevalent transition in patients treated with antiretroviral drugs.

## Introduction

The genetic diversity of HIV-1 is a subject of growing concern in regard to both diagnosis of HIV infection as well as expectations of responsiveness to antiretroviral therapy. Resistance mutations to antiretroviral drugs (ARVs) arise spontaneously as a result of the error-prone replication of HIV-1 and, in addition, are selected both in vitro and in vivo by pharmacologic pressure.[1-3] The high rate of spontaneous mutation in HIV-1 has been largely attributed to the absence of a 3'→5'exonuclease proofreading mechanism. Sequence analyses of HIV-1 DNA have detected several types of mutations, including base substitutions, additions, and deletions.[1] The frequency of spontaneous mutation for HIV-1 varies considerably as a result of differences among viral strains studied in vitro.[3] Overall mutation rates for wild-type laboratory strains of HIV-1 have been reported to range from $0.97 \times 10^{-2}$ to $2 \times 10^{-2}$ per nucleotide for HXB2 to as high as $8 \times$

$10^{-2}$ per nucleotide for the HIV-1 NY5 strain.[1-4] The rapid appearance of such mutations is, in part, a result of low fidelity during reverse transcription.

A large proportion of nucleotide substitutions that cause amino-acid changes in HIV-1 favor a guanosine-to-adenosine (G→A) transition.[5-8] The G→A transition plays an important role in viral evolution as well as in the escape of HIV-1 from the host immune response. However, the contribution of the G→A transition relative to other transitions in treated patients receiving ARVs and the generation of drug resistance mutations has not been fully assessed. To address this issue, we analyzed the rate of different nucleotide substitutions in clinical samples in the RT and PR regions.

## Materials and methods

### *Study Populations*

This study was carried out using plasma obtained from patients who were followed in our clinic from among a group of initially ARV-naive patients (n = 50) having viral loads > 1000 copies/mL. A second group included ARV-experienced patients in whom viral load was > 1000 copies/mL and in whom genotypic analysis was performed (n = 51). Plasma was obtained during 20002001. All subjects harbored subtype-B HIV-1 viruses and provided informed consent.

### *Sequencing of the RT and PR Genes*

Toward this purpose, RNA was extracted using the *QIAamp* kit and RNA products were amplified by polymerase chain reaction (PCR) as described.[9] The sequencing of DNA products was carried out by standard methodology using kits (*TruGene* ) obtained from Bayer Diagnostics Inc. (Toronto, Ontario, Canada). The sequencing of RT was limited to positions 38249 due to the type of assay performed. Sequencing of both the RT and PR genes was also employed to determine the subtypes of these viral isolates in concert with the Stanford database http://hivdb.stanford.edu/. Nucleotide genomic diversity in the RT and PR regions of the various viral isolates was compared with a consensus subtype B reference virus, LAV-1 (http://www.hiv.lanl.gov; accession number M19921).

### *Statistical Analysis*

The distribution of nucleotide substitutions was determined for each patient and the mean value for each type of substitution was calculated. Differences among types of nucleotide substitutions were determined by 1-way analysis of variance, followed by Tukey's multiple comparison test. Statistical analyses were performed using Prism software (version 3.0, GraphPad Software, Inc.).

## Results

### *Nucleotide Substitutions Among Drug-Naive Patients*

The distribution of nucleotide substitutions relative to the subtype B reference in nontreated patients is shown in Figure 1. The mean of the G→A hypermutation was 8.2 (95% confidence interval [CI], 7.39.1) compared with A→G nucleotide transitions, which was 6.5 (95% CI, 5.87.1) ($P$ < .001). This was followed by 2 other relatively frequent transitions, cytosine (C) →thymidine (T) and T→C.

### *Nucleotide Substitutions Among ARV-Experienced Patients*

To ascertain the distribution of nucleotide transitions in treated patients, we analyzed the sequences of 51 ARV-treated patients, all of whom had received nucleoside reverse transcriptase inhibitors (NRTIs) (Figure 2); the distribution of treatments in these individuals is described in the Table 1. The mean of G→A transitions was 8.1 (95% CI, 7.39), which was similar to the incidence of the A→G transition 7.7 (95% CI, 6.78.7). The mean of C→T and T→C transitions (4.2 and 3.9 mutations, respectively) was lower than that of either A→G or G→A ($P$ < .001). Among patients treated with PIs (n = 34), the mean of A→G transitions was 8.5 (95% CI, 7.49.6), which was higher than the incidence of the G→A transition 5.7 (95% CI, 4.76.7) ($P$ < .001) (data not shown). We did not analyze the data among patients treated by nonnucleoside reverse transcriptase inhibitors (NNRTIs) (n = 12) due to the fact that 8 of them had also been treated with PIs.

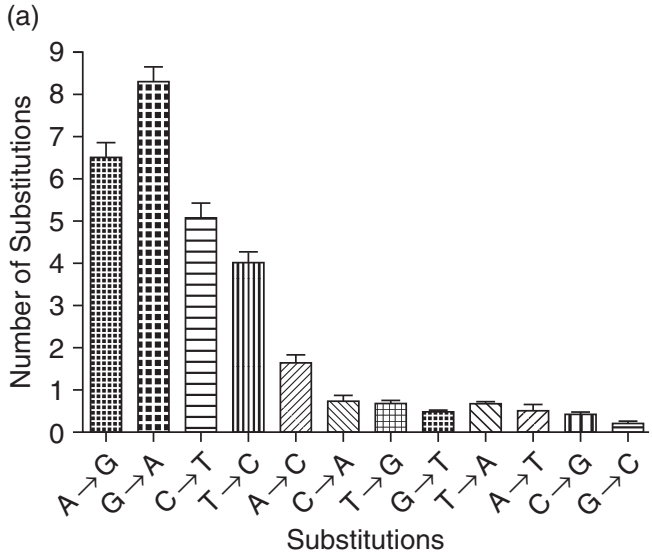


**Figure 1**
**Numbers of nucleotide substitutions in RT and PR in untreated patients**. (Values represent means for each transition between patients ± standard error of the mean).

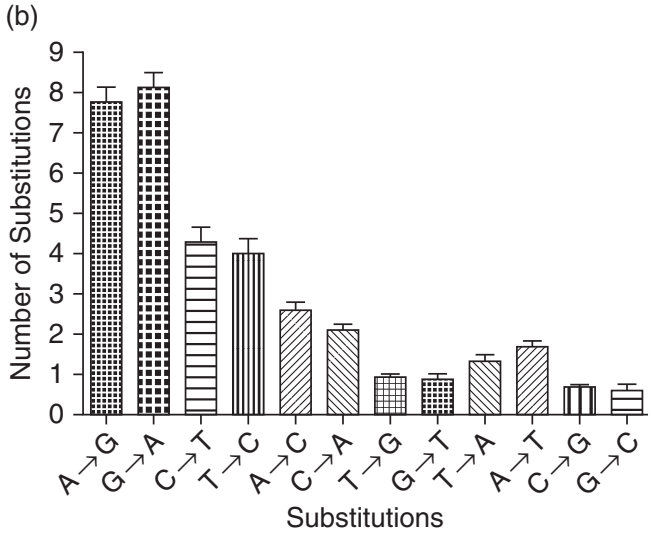


**Figure 2**
**Numbers of nucleotide substitutions in RT and PR in treated individuals**. (Values represent means for each transition between patients ± standard error of the mean).

### *Nonresistance Positions*

In order to ascertain whether the distribution of nucleotide substitutions was related to positions known to confer resistance, we conducted an analysis, which excluded all positions known to be associated with drug resistance. In the untreated group, the mean of G→A transitions, ie, 7.1 (95% CI, 6.37.9) was significantly higher than that of A→G transitions, ie, 6.1 (95% CI, 5.56.7) ($P < .05$) (Figure 3). In contrast, the mean of G→A transitions in treated patients was 3.5 (95% CI, 2.94.1), which was less than that of A→G transitions, ie, 5.1 (95% CI, 4.45.8) ($P < .01$) (Figure 4). Among patients treated with PIs, the mean of A→G transitions was 5.8 (95% CI, 56.6), which was higher than the incidence of the G→A transition, ie, 3.6 (95% CI, 2.84.4) ($P < .001$) (data not shown).

We also evaluated the prevalence of resistance-coassociated mutations (as defined by a IAS-USA consensus panel, October 2003) in relation to different nucleotide transitions in 3 groups of treated individuals, ie, patients who had received both PIs and NRTIs, both NNRTIs and NRTIs, or only NRTIs. The different regions of RT and PR were analyzed based on the types of drugs employed in therapy. Among major resistance mutations, 47% of PI-treated patients harbored the L90M mutation, which results from a T→A transversion. In contrast, only 14.7% harbored D30N and 11.7% harbored M46I, both of which result from a G→A transition.

Among all ARV-treated patients, 76.4% harbored M184V and 31.3% harbored K70R, both of which result from a A→G transition. Another high-prevalence mutation was T215Y (33.3% of patients), which is a result of both C→A and A→T transversions. Among NNRTI-treated patients, 33.3% harbored the Y181C mutation, which results from a A→G transition. G190A occurred in 25% of patients (G→C) as did V108I (G→A).

## Discussion

This study reports that the prevalence of the G→A hypermutation in treated patients was decreased compared with the prevalence in untreated patients. For convenience, we compared sequences in our patient populations with those of the LAV-1 reference virus, which is of ancestral importance. Although LAV-1 might itself have some unique sequences, this would not have affected our analysis, which compared LAV-1 isolates from both treated and untreated patients. The RT and PR enzymes are the most important targets of antiretroviral therapy, and mutations at different positions in the *pol* gene can confer resistance to different ARVs.

Some of the resistance mutations that result from a G→A transition confer only low levels of resistance when they appear alone, such as K20R and V32I in PR and D67N and G333A in RT. Other mutations resulting from G→A transitions may occur rarely, such as V82T (the preferred mutation in this position is V82A, which results from a T→C transition). In RT, V75T which confers resistance to

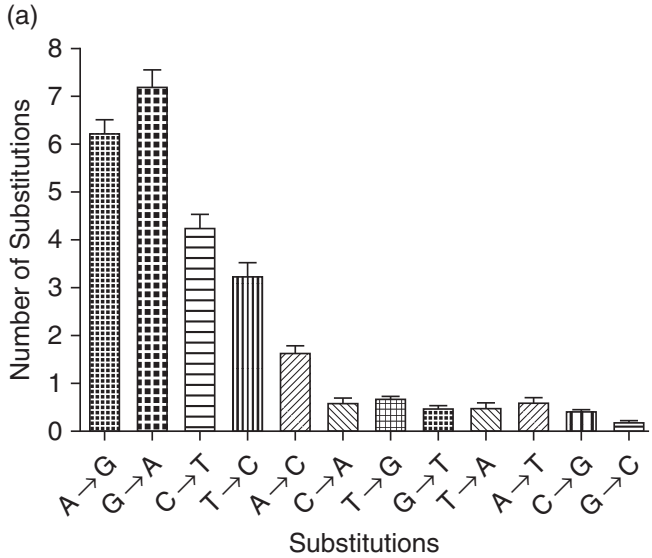


**Figure 3**
**Numbers of nucleotide substitutions in RT and PR in untreated patients, excluding positions responsible for resistance mutations as defined by a IAS-USA consensus panel, October 2003**. (Values represent means for each transition between patients ± standard error of the mean.)

**Table 1: Prevalence of Patients Harboring Different Resistance Mutations**

| Region sequenced and number of isolates examined | Nucleotide changes (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G→A | A→G | C→T | T→C | A→C | C→A | T→G | G→T | T→A | A→T | C→G | G→C |
| PR (n = 34) | K20R (2.9) | **I47V (2.9)** | L10F (0) | V82A/S **(17.6)** | I47V (0) | L10I (17.6) | L10R (5.8) | G48V **(11.7)** | **L24I (0)** | **K20M (2.9)** | **L10V (5.8)** | M46L **(0)** |
| | D30N[a] **(14.7)** | I50V **(0)** | | | I54L (2.9) | | | | **L90M (47)** | M46L **(17.6)** | I54M (2.9) | G73S (0) |
| | V32I (2.90) | I54V (14.7) | | | | | | | | | | |
| | M36I (29.4) | I84V **(11.7)** | | | | | | | | | | |
| | **M46I (11.7)** | N88D/S (8.8) | | | | | | | | | | |
| | A71T (8.8) | | | | | | | | | | | |
| | **G73S (11.7)** | | | | | | | | | | | |
| | **V77I (8.8)** | | | | | | | | | | | |
| | V82T **(5.8)** | | | | | | | | | | | |
| RT (NRTI) (n = 51) | D67N (19.6) | K65R (0) | A62V (0) | F77L (0) | M41L (21.5) | Q151M (3.9) | L74V (3.9) | V75I (0) | F116Y (1.9) | M41L (21.5) | F77G (1.9) | |
| | V75I (5.9) | K70R (31.3) | T215F (11.7) | | E44A/D (5.8) | T215Y (33.3) | L210W (25.4) | | | E44D (0) | | |
| | V118I (25) | M184V (76.4) | | | K219Q (7.8) | | | | | Q151M (3.9) | | |
| | G333A (NA) | K219E (11.7) | | | | | | | | T215F (11.7) | | |
| | | | | | | | | | T215Y (33.3) | | | |
| RT (NNRTI) (n = 12) | V108I (25) | Y181C (33.3) | P236L (0) | V106A (0) | K103N (9.6) | P225H (0) | | | L100I (8.3) | K103N (8.3) | | G190A (25) |
| | G190S (0) | Y188C (0) | | Y188H/L (16.6) | M230L (0) | | | | Y188L (9.6) | Y188L (9.6) | | |
| | | | | | | | | | G190S (0) | | | |

[a] The major mutations in PR are in bold

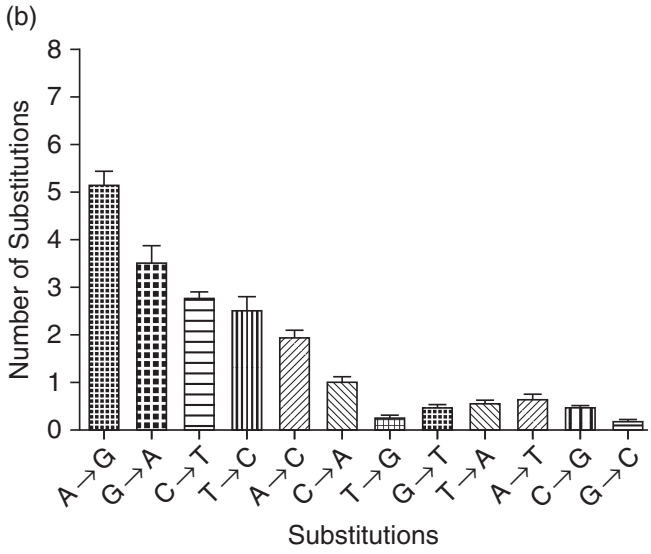


**Figure 4**
**Numbers of nucleotide substitutions in RT and PR in treated individuals, excluding positions responsible for resistance mutations as defined by a IAS-USA consensus panel, October 2003**. (Values represent means for each transition between patients ± standard error of the mean.)

stavudine only occurred in 4% of patients treated with this drug.[10] Of note, some of the common resistance mutations that are easily selected by drugs in vivo and in cell culture involve A→G transitions, eg, M184V and Y181C.

To assure that drug resistance mutation sites did not bias the total results obtained, we also analyzed the prevalence of substitutions in RT and PR while excluding codons known to be associated with drug resistance. Again, we observed a decrease in prevalence of G→A transitions and even an increased prevalence of A→G transitions.

The clinical importance of the G→A hypermutation in HIV-1 is not clear. It has been shown previously both in vitro and in vivo that the G→A nucleotide substitution is the most frequent.[11-18] In contrast, studies on intrapatient sequence variation of the *gag* gene found no differences between proportions of G→A and A→G transitions.[19]

A V106M mutation in RT is preferentially selected both in vitro and in vivo by the NNRTI efavirenz in subtype C viruses and confers high-level cross-resistance to all 3 currently approved NNRTIs.[20] The selection of this mutation in subtype C viruses results from a single nucleotide change from wild-type in subtype C viruses (GTG→ATG). The G→A hypermutation is the cause of the M184I substitution which commonly occurs prior to M184V.[21] However, M184I is rare in clinical samples and the switch from isoleucine to valine results from a A→G transition. Consideration of viral fitness or replication capacity may have an impact on the likelihood that a given substitution may ultimately prevail in cases in which several different changes may confer resistance to the same drug.[22] Sexual transmission of a HIV-1 F subtype virus that contains G→A hypermutations has been reported in 1 case, but the G→A hypermutation could no longer be detected in the transmitting patient after 1 year on ARV therapy.[23]

G→A hypermutations may involve asymmetric endogenous deoxynucleotide triphosphate (dNTP) pools, with deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP) being present at the lowest levels, while dCTP/dTTP (deoxythymidine triphosphate) ratios range between 1:2 and 1:6.[24] Thus, the G→A hypermutation in HIV has been directly linked to a dCTP pool imbalance during reverse transcription.[18,25,26] In one study, antimetabolic drugs were shown to reverse G→A hypermutations in favor of A→G transitions, by increasing the intracellular ratio of dCTP/dTTP.[27]

An alternative important cause of G→A hypermutation may involve a cellular factor, APOBEC3G, a cytidine deaminase that converts cytosine to uracil. The activity of APOBEC3G is inhibited by the Vif protein.[5,7,8] In the absence of Vif, the synthesis of the negative strand of DNA can result in the insertion of a uracil as a result of the deamination of a cytosine, leading to the inclusion of an adenosine instead of guanosine in positive-stranded cDNA. This results in mutant viruses that contain several G→A changes. With cell passage, more G→A mutations in viral DNA occur and infectivity is diminished. Furthermore, trace amounts of APOBEC3G are found within virus particles.[28] In contrast, mutant viruses that lack the *vif* gene contain higher levels of APOBEC3G. Such viruses cannot complete normal reverse transcription. This Vif-APOBEC3G interaction might explain certain cases of diminished viral fitness; hence, this interaction may be a target for future drug development.

In our descriptive study, the G→A transition was the most frequent mutation observed among untreated patients, and this may be a result of spontaneous mutation. In contrast, the G→A hypermutation was not more prevalent in treated patients than A→G transitions, and in PI-treated patients A→G was even more prevalent. Thus, patterns of nucleotide substitutions in the *pol* gene are different in treated vs untreated individuals.

Further biochemical and clinical analysis will be needed to understand the full importance of these different patterns of nucleotide substitutions in HIV-1 isolated from both treated and untreated individuals.

## Authors and Disclosures

Dan Turner, MD, has disclosed no relevant financial relationships.

Bluma Brenner, PhD, has disclosed no relevant financial relationships.

Daniela Moisi, MSc, has disclosed no relevant financial relationships.

Chen Liang, PhD, has disclosed no relevant financial relationships.

Mark A. Wainberg, PhD, has disclosed no relevant financial relationships.

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