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Impact of Human Immunodeficiency Virus Type 1 Subtype C on Drug Resistance Mutations in Patients from Botswana Failing a Nelfinavir-Containing Regimen

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Among 16 human immunodeficiency virus-infected (subtype C) Batswana patients who failed nelfinavir (NFV)-containing regimens, the most prevalent mutation observed was D30N (54%), followed by L90M (31%). L89I, K20T/I, and E35D polymorphic changes were also identified. These findings suggest that subtype C viruses in Botswana may develop resistance to NFV via subtype-specific pathways.

Human immunodeficiency virus type 1 (HIV-1) subtype C is responsible for almost half of all new infections worldwide (13). Since most current HIV-1 drugs were first studied against subtype B, it is clear that a systematic evaluation of specific pathways that may lead to resistance for each subtype is needed. In January 2002, Botswana became the first country in southern Africa to launch a national antiretroviral therapy program, making our study possible.

Previous studies demonstrated a differential evolution of drug resistance with regard to nonnucleoside reverse transcriptase inhibitor pressure through distinct mutational pathways in subtype B versus subtype C (1, 4). The current study evaluated mutations in Batswana patients who failed second-line nelfinavir (NFV)-containing regimens.

Study population. In Botswana, free AIDS-associated retroviruses are offered to individuals with CD4 counts of <200 cells/μl, and first-line therapy includes zidovidine plus lamivudine plus efavirenz (EFV) if male or zidovidine plus lamivudine plus nevirapine if female, due to potential teratogenicity associated with EFV. In cases of therapeutic failure, second-line regimens until recently included didanosine plus stavudine plus NFV.

We identified resistance mutations and polymorphisms emerging during treatment with these NFV-containing second-line regimens by performing genotyping on the first 16 cases of confirmed virological failure, i.e., viral rebound to more than 400 HIV-1 RNA copies/ml among 155 patients followed between July 2002 and May 2005. Highly active antiretroviral therapy history was available for all treated patients. Genotyping of HIV-1 *pol* genes using the Bayer Diagnostics Trugene System (San Francisco, CA), phylogenetic analysis, and confirmation of subtype C infection were performed as previously described (4). Selection of resistance to NFV using subtype B viruses as well as subtype C clinical isolates from Botswana and Ethiopia was carried out as described previously (2, 6, 10), as was statistical analysis to examine differences in polymorphisms and mutation frequencies among and between Batswana and Stanford database (SDB) isolates (4).

Of 155 HIV-1 subtype C-infected patients who had started on an NFV-containing regimen, 16 showed evidence of virologic failure, and all had been protease inhibitor (PI) naïve at the initiation of NFV-based therapy. CD4 cell counts and measurement of plasma viral loads were also available from times prior to treatment initiation (day 0), at time of first failure, before we switched to a second-line regimen, and at second failure or time of switch to a third regimen, and the median duration of each nucleoside reverse transcriptase inhibitor/nonnucleoside reverse transcriptase inhibitor or NFV-based regimen was 12 months. Although these patients improved with regard to the average CD4 cell counts (means ± standard deviations), from 95.8 ± 35.7 cells/μl (pretherapy baseline) to 251.6 ± 45.9 cells/μl by the end of second-line therapy, most of them (12 of 16) did not have significant reductions in plasma viremia. The average viral loads at baseline, first failure, and second failure were 281×10^3, 90×10^3, and 59×10^3 HIV-1 RNA copies/ml, respectively.

Frequency of mutations and polymorphisms in protease (PR) of NFV-treated patients. The time after NFV initiation at which *pol* genes were sequenced was 12.58 ± 4.25 months (mean ± standard deviation). Three of the 16 patients with virological failure did not develop any significant NFV-associated mutations. Eight of the 10 known NFV resistance-associated mutations (9) were identified in 13 of these 16 individuals as follows: M36I (92.3%), D30N (54%), N88S/D (38.5%), L33F (31%), L90M (31%), M46I (23%), L10F (23%), and A71V (15%). Several minor mutations that have a minimal impact on phenotypes and several polymorphisms that are naturally occurring variations that may or may not have an

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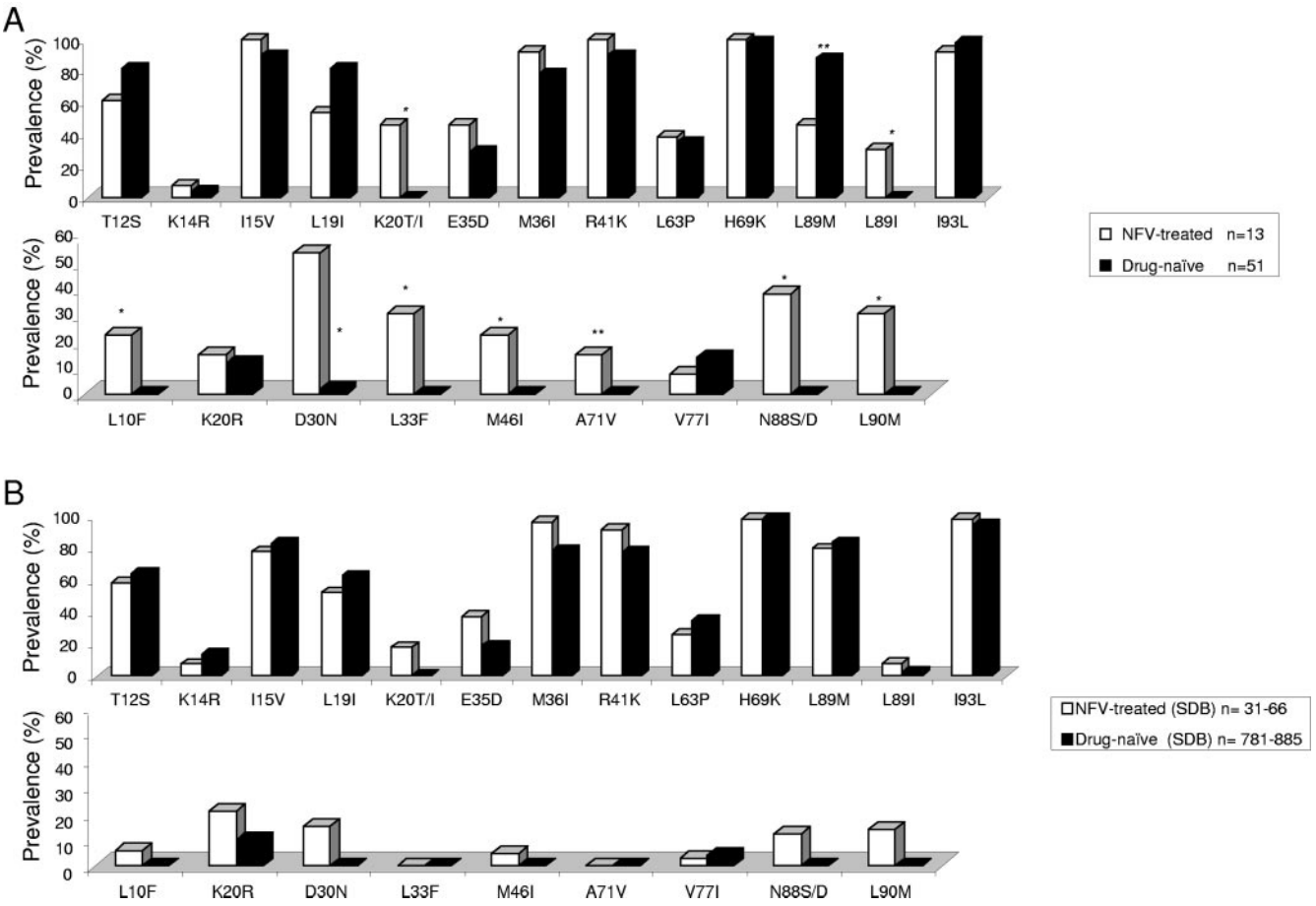

FIG. 1. Prevalence of HIV-1 subtype C protease mutations and polymorphisms in Batswana patients receiving nelfinavir. Comparison with HIV-1 subtype C Stanford database. Polymorphisms and mutation frequencies at amino acid positions 1 to 99 of PR sequences from Batswana (A) and SDB (B) HIV-1 subtype C-infected patients. (A) Sequences from the 51 Batswana patients at baseline (black bars) and the 13 Batswana patients presenting primary mutations associated with NFV resistance (white bars) (B) SDB sequences pretherapy (black bars) and SDB sequences from 31 to 66 NFV-treated patients (white bars). Statistical significance was determined by Fisher's two-tailed exact test. *, $P < 0.001$; **, $P < 0.05$.

impact on levels of drug resistance were also found among NFV-treated and drug-naïve Batswana patients (Fig. 1A).

No major differences were found between 51 Batswana drug-naïve (12) and NFV-treated patients ($n = 13$) with regard to polymorphism profile, except that L89M occurred in 46% of the NFV-treated patients versus 88% of drug-naïve patients ($P = 0.003$). Interestingly, L89I was never detected in sequences from PI-naïve Batswana patients but occurred in 31% of NFV-treated patients ($P = 0.001$). Compared to HIV-1 subtype C sequences from the SDB (Fig. 1B), D30N was the most prevalent mutation among Batswana sequences (54% versus 15.3%, $P = 0.0056$). Interestingly, many mutations associated with NFV resistance in Batswana patients were not present or emerged at low frequency in HIV-1 subtype C sequences from the SDB. The SDB subtype C sequences are mostly of Ethiopian origin, and the higher rate of selection of D30N in the Batswana patients could be due to different adherence/exposure levels and/or the duration of treatment.

Indeed, L89M was seen more frequently in the SDB NFV-experienced population than in the equivalent Batswana cohort (80% versus 46%), whereas L89I was more frequent in Batswana NFV-experienced patients than in SDB equivalent sequences (31% versus 8.3%). We also examined the profiles of mutations and natural polymorphisms among the 13 Batswana NFV-treated patient sequences (Table 1) and found in comparisons of 8 of 13 sequences from before and after NFV treatment that numerous polymorphisms, such as L10M, V11F, I13V, K14R, K20T/I, E35D, K45Q, D60E, Q61E, I62V, I64V, and L89I, were acquired during NFV exposure. The K20T polymorphic change was observed in 4 of 13 sequences in the presence of L89M/I (Table 1). The number of polymorphic changes observed following NFV exposure was inversely proportional to the number of mutations associated with NFV resistance (data not shown).

In vitro selection of NFV-resistant variants. Viruses of diverse origins (Botswana, BG-5, BG-15, and HB-1; Ethiopia, 4742 and 4761; subtype B, 4246 and 3350) were passaged at increasing NFV concentrations (0.005 μM to 10 μM) in cord blood mononuclear cells; all harbored the D30N primary mutation at the end point of the selection period of 30 weeks (Table 2). Several previously reported amino acid changes were also noted in the subtype C isolates, e.g., M46I and L10F, and in addition, numerous natural polymorphisms were found in the PR gene of subtype C compared to that of subtype B.

TABLE 1. PR polymorphism profiles of NFV-treated patients presenting with mutations[a]

Patient	Mutation(s)	Polymorphisms
1*	D30N	T12S, G16A, L19I, **E35D**, **L89I**/M, C67E, **K20T**, **K45Q**, D60E
2	A71V, L90M	T12S, I13V, L19V, K20T, E35G, K70R, T74S, V82I, L89I
3	D30N, L63P	T12S, L19V, E35D, K45Q, Q61E, V75I, K20T, D60E
4*	K20R, L90M	**K14R**, L19I, **K20T**, **E35D**, **D60E**, **Q61E**, **I64V**, L89M
5	L10F, N88S	L19I, K20T, E35D, Q61H, L63T, T74S, L89M
6*	M46I, L10F, N88S	T12S, L19V, **K20I**, L63V, **I64V**, T74V, L89M
7*	D30N, K20R, L33F	**L10M**, **V11F**, **I13V**, E35D, **I62V**, L63T
8	D30N, L33F, N88D	T12S, G16E, L19I, M36L, L63Q, L89I
9*	D30N, N88D, L33F, L63P	T12S, L19I, I62V, T74A, **L89I**/M
10	D30N, L90M, L33F, A71V, L63P	G17D, L19V, E35D, M36L, S37K
11*	M46I, V77I, L90M	T12S, L19I, L63V, L89M
12*	N88S, L63P	T12S, L19I, I64L
13*	D30N, N88D, M46I, L10F, L63P	

[a] Polymorphisms V3I, I15V, S37N, R41K, H69K, I93L, and the M36I minor mutation were common to all of these patients, except for patient 8 who did not harbor M36I, S37N, or I93L. The asterisk indicates patients for whom pre-NFV sequences were available. Polymorphic changes occurring during NFV treatment appear in bold.

Thus, the preferential selection of D30N among Batswana patients was confirmed by in vitro selection of NFV resistance using Batswana clinical isolates. Analysis of 33 available Ethiopian sequences in the SDB shows that only 12 contained mutations associated with NFV resistance. A comparison of these mutated sequences with our Batswana mutated sequences revealed that the frequencies of the D30N mutation were similar in both the Batswana and Ethiopian populations (3).

As stated, the low frequency of D30N in Ethiopian patients may reflect a lower proportion of failing patients presenting with major NFV-associated mutations in that group (8). Although D30N is preferentially selected with NFV in Botswana, L90M variants may arise as minority species in selected patients, e.g., patient 10. Among four Batswana patients presenting with the L89I polymorphic change, three also carried the D30N mutation and one carried L90M, suggesting that L89I may facilitate both the D30N and L90M pathways leading to NFV resistance. We also observed the L33F and N88D/S mutations in the context of D30N in our Batswana patients. N88D/S is associated with reduced NFV susceptibility (14, 18). It has also been shown that N88D and L89M may compensate for a loss of viral replicative capacity mediated by D30N (15).

We have previously shown that 73% of PI-naïve Batswana patients exhibited more than two minor mutations, mainly K20R, M36I, V77I, and L63P, associated with PI resistance (4). Although L63P alone has no effect on NFV susceptibility, it has been shown to restore replicative fitness in combination with other minor mutations (16, 18). L63P is also classified as a minor mutation associated with lopinavir resistance (9). A recent in vitro study demonstrated hypersusceptibility to lopinavir in subtype C viruses, providing evidence that subtype-specific polymorphisms (I93L) can play a role in drug activity (7). These data are important considering the relatively high frequencies of L63P (40%) and I93L (97%) among Batswana drug-naïve patients and considering that Botswana has recently elected to use ritonavir-boosted lopinavir in place of NFV in second-line regimens.

The presence of M36I, R41K, H69K, and L89M in subtype C is associated with increased PR catalytic activity (17). It is interesting that the presence of D30N as a sole major mutation associated with NFV resistance occurred in the context of a high background of natural polymorphisms and polymorphic changes, whereas an accumulation of major and/or minor mutations was observed in viruses containing only a few natural polymorphisms (Table 1). Others have proposed that the accumulation of nonactive site polymorphisms not only is limited to a compensatory role with regard to D30N impairment of viral replication but also may be responsible for a loss in binding affinity of PIs due to enzymatic geometric distortions (11). In addition to resistance mutations, we also showed that polymorphic changes, such as K20T/I, occurred during NFV treatment (Fig. 1). Interestingly, K20T has previously been associated with an increase in NFV susceptibility (18).

There is a consensus concerning the comparable response to highly active antiretroviral therapy in subtype B- versus non-

TABLE 2. Selection of viruses resistant to NFV using HIV-1 subtype C clinical isolates

Virus[a]	Country	Subtype	Mutations	Polymorphism(s)[b]
4246	Canada	B	*D30N, *V77I	T12K*, I93L*
3350	Canada	B	*D30N, *M46I, L63P, V77I	I93L
BG-5	Botswana	C	*D30N, *L10F	T12S, *I13V, I15V, L19I, M36I, R41K, L63V, H69K, L89M, I93L
BG-15	Botswana	C	*D30N, *M46L	T12S, *I13V, I15V, L19I, R41K, V77I, L89M, I93L
HB-1	Botswana	C	*D30N, *M46I, L63P, *V77I	T12S, *I13V, *I15V, L19I, *L23I, M36I, R41K, *I62V, H69K, L89M, I93L
4742	Ethiopia	C	*D30N, *M46I	I13V*, K14R, I15V, M36I, R41K, H69K, T74S, L89M, I93L
4761	Ethiopia	C	*D30N, *M46I	T12S, I15V, *K20T, M36I, R41K, Q61H, H69K, *L89I, I93L

[a] Nucleotide accession numbers: BG-5, AF492600; BG-15, AF492601; HB-1, AF492607; 4742, AF492595; 4761, AF492597; 4246, AY213138; and 3350, AY236362.
[b] *, mutations and polymorphic changes occurring under NFV pressure.

subtype B-treated patients (5). However, vigilance is necessary with regard to evidence of subtype-specific pathways for resistance which may affect interpretation of results. Finally, the presence of both resistance mutations and polymorphisms confirms the desirability of having access to pre- as well as post-therapy resistance testing in developing country settings.

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