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Variants Other than Aspartic Acid at Codon 69 of the Human Immunodeficiency Virus Type 1 Reverse Transcriptase Gene Affect Susceptibility to Nucleoside Analogs

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The T69D mutation in the human immunodeficiency virus type 1 reverse transcriptase (RT) gene has been associated with reduced susceptibility to dideoxycytosine (ddC); however, several other mutations at codon 69 have been observed in antiretroviral drug-treated patients. The Stanford HIV RT and Protease Sequence Database was interrogated and showed that 23% of patients treated with nucleoside RT inhibitors (NRTI) had mutations at codon 69. These variants included T69N, -S, -A, -G, -E, -I, and -K mutations that were present in patients treated with NRTI but not in drug-naive patients. Treatment history information showed that a substantial percentage of these codon 69 changes occurred in patients administered non-ddC-containing regimens. Different and specific patterns of other RT gene mutations were associated with the various codon 69 mutations. Drug susceptibility assays showed that viral constructs containing codon 69 variants could have reduced susceptibility to ddC and other RT inhibitors. These results suggest that the T69D mutation is not the only codon 69 variant associated with drug resistance and that ddC is not the only drug affected.

Nucleoside reverse transcriptase inhibitors (NRTI) are an important component of successful antiretroviral therapy. Combinations of two or more NRTI with protease inhibitors and/or nonnucleoside reverse transcriptase inhibitors (NNRTI) are currently the standard of care for the treatment of naive and antiretroviral drug-experienced individuals (5). Most patients, however, eventually show evidence of waning antiviral activity, as measured by increases in virus levels in plasma. Mutations in the protease and/or reverse transcriptase (RT) gene are typically evident at this time through genotyping assays (10). Many mutations in the RT gene have been associated with reduced susceptibility to NRTI (17). Several of these mutations arise in the β3-β4 loop of the human immunodeficiency virus type 1 (HIV-1) RT enzyme (20). Specific amino acid changes at codons 65, 67, 69, 70, and 74 confer reduced susceptibility to one or more NRTI (17). These mutations directly cause or contribute to reduced susceptibility through mechanisms such as repositioning of the primer-template complex (4), increasing the enzyme's selectivity for deoxynucleoside triphosphates over dideoxynucleoside triphosphates (20), and enhancing pyrophosphorolytic activity (1).

A mutation at codon 69 from threonine to aspartic acid has been shown to confer resistance to dideoxycytosine (ddC) (9). Recently, two amino acid insertions after codon 69 have been shown to confer resistance to nearly all NRTI alone or in combination with other RT gene mutations (7, 14, 21). Physician-requested genotyping has also revealed other mutations at codon 69 which have not yet been defined. In this report, the prevalence of codon 69 mutations was examined and the susceptibility of these variants to NRTI was studied.

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MATERIALS AND METHODS

Database. The frequency of different mutations at codon 69 was examined through the Stanford HIV RT and Protease Sequence Database (http://hivdb.stanford.edu) (11). This relational database contains approximately 15,000 published HIV RT sequences obtained from GenBank, journal articles, and international collaboration databases. The antiretroviral treatment history and source of each isolate are also housed in the database. Sequences from approximately 1,100 clade B NRTI-treated patients were used in this study. 25% had received one NRTI; 40% had received two NRTI; 11% each had received three, four, and five NRTI; and 3% had received six or more NRTI. Standard browser-driven database queries were used to access and tabulate most mutation data. However, in some instances, beta-test versions of queries (kindly provided by Robert Shafer) were used. Some patients were excluded from certain analyses when treatment information was not appropriately defined (e.g., some patients were known to be NRTI experienced, but the exact NRTI taken were not available). Mutation frequency analyses were restricted to include only one sequence per patient; when multiple sequences for a given patient were in the database, the sequence after the longest duration of therapy for that patient was used. Statistical differences were determined by using chi-square or Fisher's exact tests, where appropriate.

Susceptibility assay. Virus constructs with various substitutions at codon 69 were created by site-directed mutagenesis on pNL4-3, and virus stocks were created by homologous recombination (21). SupT1 cells were infected with 30 to 100 tissue culture infective doses of virus for 1 h at 37°C and then washed to remove nonbound virus. Virus-infected cells (100,000) were dispensed into 96-well plates containing six fourfold dilutions of drug in triplicate. After 4 days, p24 antigen levels in the culture supernatant were measured by enzyme-linked immunosorbent assay (NEN Life Sciences) and the amount of drug required to inhibit viral replication by 50% was calculated. Each isolate was tested a minimum of three times against each drug. Significant differences in drug susceptibility between the NL4-3 control isolate and the codon 69 variants were determined using unpaired *t* tests.

RESULTS

Mutation frequency. The percentages of patients with zidovudine (AZT), dideoxyinosine (ddI), ddC, 2′,3′-didehydro-3′-deoxythymidine (d4T), or β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) use in their treatment histories were 70, 44, 20, 30, and 44, respectively. For patients treated with four or more NRTI, 100% used AZT, 83% used ddI, 63% used ddC, 95%

TABLE 1. Frequency of resistance-associated RT gene mutations in isolates from NRTI-treated patients[a]

Codon	% of isolates with mutation		*P* value
	1 to 3 NRTI	≥4 NRTI	
41	29	63	<0.0001
65	3	3	>0.9
67	21	64	<0.0001
69	15	34	<0.0001
70	28	32	0.38
74	7	17	0.0005
75	5	17	<0.0001
184	29	55	<0.0001
210	21	50	<0.0001
215	43	76	<0.0001
219	17	38	<0.0001

[a] The isolates were from 638 patients given one to three NRTI and 121 patients given four or more NRTI.

used d4T, and 99% used 3TC. Table 1 shows the prevalence of major RT mutations in patients treated with NRTI. Nearly all mutations were significantly more prevalent in heavily treated patients than in patients treated with one to three NRTI. Mutations at codon 69 were less frequent than changes at codons 41, 67, 70, 210, and 215 that are typically associated with AZT resistance (3, 12). However, codon 69 changes were more prevalent than changes at codons 65, 74, and 75 (Table 1) despite the fact that ddC was the least-prevalent NRTI used during treatment. While codon 69 mutations were more frequently found in patients who received ddC treatment than in patients who never received ddC (48 of 137 patients with ddC treatment versus 77 of 492 patients without ddC treatment [$P < 0.001$]), a substantial proportion (15%) of ddC-naive patients possessed codon 69 mutations.

Codon 69 variants. Table 2 shows the distribution of the specific amino acid mutations at codon 69. No mutations at codon 69 were found in treatment-naive patients. Twenty-one percent of patients who had received NRTI therapy had a mutations at codon 69. Only one patient had received ddC monotherapy, and 4.7% of the isolates were from patients who received only AZT-ddC combination therapy. The T69D and T69N mutations were the most frequently observed mutations, and the T69D mutation was found more frequently in heavily treated patients ($P < 0.001$). The mutations T69A, -G, -I, -E, and -K were rare and distributed among lightly and heavily treated patients.

Other RT mutations. The association of other RT gene mutations with the codon 69 changes in NRTI-treated patients is shown in Table 3. Compared to NRTI-treated patients without codon 69 changes, patients with T69D mutations had significantly higher frequencies of M41L, D67N, K70R, V75I/A/M/T (V75 to I or A or M or T), M184I/V, L210W, T215Y/F, and K219E/Q ($P \leq 0.0008$). Patients with T69N mutations had higher frequencies of D67N, K70R, M184I/V, and T215F than patients with wild-type codon 69 did ($P < 0.0001$) and had lower frequencies of M41L ($P = 0.002$), L210W ($P < 0.0001$), and T215Y ($P < 0.0001$). Patients with T69S or T69A were significantly more likely to have a RT gene insert or deletion ($P < 0.0001$). All patients with T69I had the K65R mutation, and four of five patients had the Q151M mutation ($P < 0.0001$ compared to patients without codon 69 changes).

Drug susceptibility. Table 4 shows the results of these assays as fold change compared to NL4-3. All constructs except T69G showed reduced susceptibility to at least one NRTI. The T69D construct showed reduced susceptibility to ddC in concordance with the results of a previous report (9). The T69N construct showed reduced susceptibility to AZT, ddI, and ddC, while the T69A construct was less susceptible to AZT. None of the constructs showed a significant change in susceptibility to either 3TC or d4T.

NRTI regimens selecting codon 69 variants. Treatment regimens from which more than one patient emerged with a codon 69 mutation are listed in Table 5. AZT was the most prevalent NRTI found in this analysis, which is consistent with the fact that AZT was the most prevalent NRTI used among all patients in the database. AZT monotherapy and AZT-3TC combination therapy were found to select for T69D, T69N, and T69S mutations in more than one patient.

DISCUSSION

The results presented here indicate that codon 69 mutations have a larger role in NRTI resistance than currently thought. The T69D mutation and its association with ddC resistance were originally presented by Fitzgibbon et al. (9). While the existence of the T69N mutation in NRTI-treated patients has been mentioned previously (18), current literature and genotype interpretation guidelines associate codon 69 only with ddC resistance. While our database analysis indicates that codon 69 changes are associated with ddC treatment, a substantial proportion of patients develops codon 69 changes without ddC experience. Also, codon 69 changes appeared at only a slightly lower frequency than those of several other NRTI-associated mutations, even though ddC was used by fewer patients than those using other NRTI. These data suggest that codon 69 changes may be selected by or maintained during treatment histories that do not include ddC.

The database analysis showed some significant associations of codon 69 variants with other RT gene mutations. The T69N

TABLE 2. Amino acid variation at codon 69 of the HIV-1 RT gene[a]

Amino acid at codon 69	% of isolates with amino acid		
	Nontreated	1 to 3 NRTI	≥4 NRTI
T	100	84.5	66.1
D	0	5.5[b]	24.1[b,c]
N	0	4.2[b]	7.4[b,d]
S	0	3.3[e]	0.0
A	0	1.0	0.8
G	0	0.1	0.0
E	0	0.1	0.0
I	0	1.0	0.8
K	0	0.0	0.8

[a] The isolates were from 267 nontreated (control) patients, 638 patients given one to three NRTI, and 121 patients given four or more NRTI.
[b] Significantly different from the values obtained with the nontreated group ($P < 0.0001$).
[c] Significantly different from the values obtained with the group given one to three NRTI ($P < 0.0001$).
[d] Significantly different from the values obtained with the group given one to three NRTI ($P = 0.03$).
[e] Significantly different from the values obtained with the nontreated group ($P = 0.001$).

TABLE 3. Frequency of RT gene mutations associated with codon 69 mutations in NRTI-treated individuals

Mutation	No. of patients	% of patients with RT gene mutation												
		41L	65R	67N	Ins/Del[a]	70R	74V	75I/A/M/T	151M	184I/V	210W	215Y	215F	219Q/E
T69D	86	64[b]	0	69[b]	0	43[b]	1	12[c]	3	57[b]	47[b]	56[b]	28[b]	32[b]
T69N	57	15[d]	0	46[b]	0	90[b]	0	0	3	46[b]	0[b]	4[b]	39[b]	64[b]
T69S	28	44	0	11	57[b]	39	0	0	0	27	23	50	0	0
T69A	12	62	0	15	17[b]	0	15	0	0	54	46	62	0	15
T69I	5	0	100[b]	0	0	80	0	80[b]	80[b]	40	0	0	0	0
T69E	3	33	0	100	0	66	0	0	0	0	33	33	33	66
T69G	2	100	0	100	0	50	0	50	0	50	0	0	50	50
T69K	1	0	0	0	0	0	0	100	100	100	0	0	0	0
None (wild type)	896	35	1	19	0	23	5	3	3	35	20	35	4	8

[a] Ins/Del, insertion or deletion.
[b] Significantly different from the values obtained with patients with wild-type codon 69 ($P \leq 0.0001$).
[c] Significantly different from the values obtained with patients with wild-type codon 69 ($P = 0.0008$).
[d] Significantly different from the values obtained with patients with wild-type codon 69 ($P = 0.002$).

mutation was found in the database analysis to be less likely to be associated with M41L, L210W, and T215Y. These data suggest that this mutation may be sufficiently contributing to AZT resistance with D67N, K70R, and T215F without requiring the mutations generally required to generate high-level AZT resistance (i.e., M41L, L210W, and T215Y) (12). While the changes in drug susceptibility in this study were small, recent studies have shown that even relatively modest susceptibility changes can correlate with clinical outcome (6, 16).

Two other significant mutational associations were seen with codon 69 mutations. The T69I mutation was always associated with the K65R mutation and almost always found with the Q151M mutation. However, not all patients with Q151M have the T69I mutation. The T69I mutation may be a requirement for these specific HIV strains to overcome spatial constraints in the RT enzyme carrying other mutations and polymorphisms. Alternatively, the T69I mutation may be involved in modulating susceptibility to one or more NRTI. Over half of the T69S mutations were primarily found in insert- or deletion-containing strains. Patients with T69S but without an insertion or deletion had mutation frequencies similar to those of NRTI-treated patients with wild-type codon 69 (data not shown). Again, this codon 69 variant may have an impact on enzyme fitness in the presence or absence of inserts or deletions and/or contribute to reduced susceptibility to NRTI alone or in association with other mutations.

In vitro susceptibility data showed that codon 69 variants had reduced susceptibility to NRTI. No other studies have directly studied point mutations at codon 69 other than T69D (9). These results indicate that codon 69 changes can impact drug susceptibility beyond ddC in an otherwise wild-type setting. Some mutations have been shown to modulate susceptibility conferred by other mutations (15, 19, 21). Most occurrences of codon 69 mutations were with other NRTI muta-

TABLE 4. Susceptibilities of codon 69 mutants to nucleoside analogs

Construct	Fold change in susceptibility compared to NL4-3				
	AZT	ddI	ddC	3TC	d4T
T69D	1	3	8[a]	1	1
T69N	7[a]	5[a]	8[a]	1	3
T69S	2	3	4[a]	1	2
T69A	5[a]	1	1	1	2
T69G	1	1	1	1	1

[a] Significantly different from the values obtained with NL4-3 ($P < 0.01$).

TABLE 5. Treatment regimens of patients with codon 69 mutations

Mutation	Patients with defined history/total no. of patients[a]	No. of patients with mutation after regimen	Treatment regimen
T69D	39/86	5	AZT monotherapy
		5	AZT then AZT + ddI
		5	AZT + 3TC
		4	AZT + ddI
		3	AZT, then AZT + ddC
		2	AZT + ddI, then AZT + 3TC
		62	Multiple[b]
T69N	36/57	11	AZT monotherapy
		10	AZT + 3TC
		4	AZT, then ddI
		4	AZT, then AZT + ddI
		3	3TC monotherapy
		25	Multiple
T69S	16/28	3	AZT + 3TC
		3	ddI, then AZT + ddI
		2	AZT monotherapy
		2	AZT, then AZT + ddC
		18	Multiple
T69A	10/12	12	Multiple
T69I	4/5	2	AZT + ddI
		3	Multiple
T69E	2/3	3	Multiple
T69G	2/2	2	Multiple
T69K	1/1	1	Multiple

[a] Patients with defined history are those for which the exact sequence and combinations of drugs are known; others are known to have NRTI experience, but the precise sequence and/or combinations are not known.
[b] Multiple regimens include single occurrences of patients that received three or less drugs, patients that received more than three drugs in more than three regimens, and patients with confirmed but undefined NRTI experience.

tions. Our laboratory work did not examine the interaction of other mutations with the codon 69 changes in susceptibility assays, for example, the impact of codon 69 variants on AZT susceptibility in the presence of AZT resistance mutations and M184V/I. The large number of possible mutation combinations and associated polymorphisms makes this analysis best suited for large phenotypic assay databases containing results from clinical isolates. These databases would be able to genotypically match large numbers of isolates except for the codon 69 mutations and statistically analyze the difference between drug susceptibility in the presence or absence of codon 69 changes. This "virtual phenotype" analysis is currently being applied in research and clinical settings (13).

The treatment regimens that select for the different codon 69 mutations were examined and reported (Table 5). AZT monotherapy was a relatively common regimen selecting for codon 69 changes, as was AZT-3TC combination therapy. These observations could not be statistically validated, as a majority of patients had extensive and complicated treatment histories of varied duration that prevent treatment groups of reasonable numbers from being assembled. While the total amount of data is quite large, some treatment regimens are underrepresented. These factors limit the ability of database information to significantly address certain questions; however examination of such data can be useful to identify hypotheses that can be evaluated in well-controlled trials or data sets.

The results presented here suggest that codon 69 changes have a wider impact on NRTI resistance than currently thought. In clinical isolates, codon 69 changes develop and persist across a wide range of treatment regimens. To date, codon 69 mutations have not been reported to directly affect clinical outcome. Since the Stanford database does not collect viral load or CD4 measurements on patients, such analysis could not be performed in this report. However, the results presented here suggest that genotypic analysis of clinical outcome in randomized trials should include all variants of codon 69 and not restricted to T69D. While current understanding of the relationships between genotype, resistance, and clinical outcome has been shown to be beneficial for patient management (2, 6, 8), further enhancement of this knowledge will undoubtedly increase the long-term efficacy of antiretroviral treatment regimens.

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