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Subtle Decreases in Stavudine Phenotypic Susceptibility Predict Poor Virologic Response to Stavudine Monotherapy in Zidovudine-Experienced Patients

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Summary: To identify the level of phenotypic susceptibility for stavudine (d4T) that is associated with a diminished virologic response to d4T therapy, phenotyping was performed on archived baseline HIV isolates from 26 subjects who received d4T monotherapy in AIDS Clinical Trials Group (ACTG) 302 who had received >3 years of prior zidovudine (ZDV) monotherapy. Seven of 26 subjects achieved a virologic response of >0.3-$\log_{10}$ copies/mL reduction in plasma HIV RNA after 8 weeks of d4T. Responders had lower fold changes in susceptibility to d4T (1.0 vs. 1.6, $p = .003$), lower baseline viral loads (4.26 vs. 4.74 $\log_{10}$ copies/mL, $p = .004$), and fewer thymidine analog mutations (TAMS) (1 vs. 2, $p = .059$). Lower baseline d4T fold change in susceptibility predicted greater reductions in HIV RNA from baseline to week 8 after adjusting for baseline HIV RNA, ZDV fold change in susceptibility, and number of TAMS. Using the same phenotypic assay, drug susceptibility among 240 antiretroviral-naive patients found all HIV isolates to have d4T susceptibility ≤1.4-fold change. Using ≤1.4 as the d4T cutoff, the positive predictive value for a virologic response in this study was 44%, and the negative predictive value was 100%. d4T susceptibility greater than 1.4-fold change was associated with failure to achieve significant viral load reduction after 8 weeks of d4T monotherapy. **Key Words:** Stavudine—Zidovudine—Drug resistance—Phenotypic susceptibility.

There is increasing evidence of cross-resistance between the thymidine analogs stavudine (d4T) and zidovudine (ZDV). d4T and ZDV therapy both select for the thymidine analog mutations (TAMS) at positions M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E/N of the HIV-1 reverse transcriptase (RT) (1–6). Despite selection of similar mutational patterns and diminished antiviral activity of both drugs in patients whose viruses contain some or all of these mutations (7–9), only small changes in drug susceptibility to d4T are observed in phenotypic assays among viruses containing TAMS. The highest levels of d4T resistance observed in phenotypic assays are generally less than 20-fold, and the vast majority of isolates with TAMS from treatment-experienced patients are less than fivefold.

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The susceptibility of virus isolates or recombinant viruses is reported either as "fold-change in susceptibility" comparing the drug IC_{50} (concentration of drug required to inhibit viral replication by 50%) of the patient's virus isolate or recombinant HIV vector containing the patient's RT and protease gene, to the IC_{50} of a wild-type reference virus. The cut-off values for susceptibility in these assays were originally based on the reproducibility of the entire assay (2.5 for PhenoSense and 4.0 for the Antivirogram) and not on data linked to virologic response. More recently, clinically derived cutoffs have been determined for abacavir (10), tenofovir (11), and lopinavir/ritonavir (12), based on the lowest level of drug resistance that is associated with a reduced virologic response to therapy in clinical trials. Clinically derived cutoffs for the rest of the antiretrovirals are needed to assist in the appropriate selection of antiretroviral therapies in antiretroviral-experienced patients.

The goal of this study was to define a clinically determined cutoff of d4T phenotypic susceptibility in a group of patients who received d4T as a single drug after ZDV treatment in a clinical trial performed during the era of antiretroviral monotherapy.

METHODS

Study Design

AIDS Clinical Trials Group (ACTG) 302 study was a randomized placebo-controlled trial that began in 1995 (13) and included patients who had previously participated in ACTG 175 (14). For this study, eligible patients were those who had received ZDV monotherapy during their participation in ACTG 175 and were randomized to subsequently receive treatment with d4T in ACTG 302 (13). Forty-six patients met the eligibility criteria. Phenotypic drug susceptibility testing was performed on baseline samples from 26 of the 46 patients for whom study baseline and week 8 plasma samples were available. Genotypic analysis performed on the same samples has been reported previously (9).

Resistance Testing

Phenotyping

Drug susceptibility was measured by the PhenoSense HIV assay (ViroLogic, S. San Francisco, CA) (15) on archived plasma samples stored at −70°C. Briefly, protease (PR) and reverse transcriptase (RT) coding sequences are amplified by reverse transcriptase polymerase chain reaction (RT-PCR) assay and cloned into a recombinant HIV vector, which also contains a luciferase reporter gene using restriction endonucleases *Apa*I and *Pin*AI to generate resistance test vectors (RTVs). The RTVs are transfected into 293 cells; virus is harvested and used to infect fresh 293 cells in the presence and absence of drug. The concentration of drug required to inhibit viral replication by 50% (IC_{50}) in the single cycle assay is determined and compared with the IC_{50} simultaneously tested, drug-sensitive reference strain containing PR and RT coding sequences from strain NL4-3. The reported values are the IC_{50} and the "fold-change" in the IC_{50}, which represents the ratios of the patient's RTV IC_{50} to the wild-type NL4-3 reference IC_{50}.

Phenotyping was performed twice on 21 of the 26 samples to assess reproducibility of d4T susceptibility in this study. The coefficient of variation of d4T fold changes from previous PhenoSense assay validation studies was approximately 10% (16).

Genotyping was performed using a direct method (Perkin-Elmer ABI, Foster City, CA) as described previously (9). Roche Amplicor (Roche Diagnostics, Indianapolis, IN) was used to quantify HIV RNA at baseline and after 8 weeks of d4T therapy.

Statistical Analyses

Virologic response was defined as a reduction of >0.3 $\log_{10}$ copies/mL from baseline at week 8 of d4T therapy. All others were considered nonresponders. A 0.3 $\log_{10}$ copies/mL cutoff was set before collecting the data because this has been considered to be the range of variation of Roche Amplicor viral load assay and of sequential viral load determinations in individual patients not on therapy (17). The geometric mean of the two d4T susceptibilities was used for analyzing 21 patients who had phenotypic assays performed in duplicate. Medians and frequencies were calculated to summarize baseline characteristics for all patients. The Fisher exact and the exact Wilcoxon tests were used to compare the characteristics of responders and nonresponders at study entry. Correlation coefficients were determined using the Spearman rank method. Linear regression was used to evaluate the association between quantitative change in HIV RNA from study entry to week 8, and the $\log_2$ d4T fold change in susceptibility adjusted for the baseline $\log_{10}$ HIV RNA level, the $\log_{10}$ ZDV fold change in susceptibility, and the number of TAMS. Log base 2 was used to give estimates of association for d4T susceptibility that are meaningful given the limited range of d4T fold changes found in this study. *P* values were all two-sided. Statistical analyses were done using SAS (SAS Institute, Cary, NC) and StatXact (Cytel Corp., Cambridge, MA)

RESULTS

The median change in plasma HIV RNA from baseline to week 8 in the 26 subjects was a reduction of 0.14 $\log_{10}$ copies/mL. Seven of the 26 subjects had reductions greater than 0.3 $\log_{10}$ copies/mL and were considered virologic responders (Table 1). Table 2 shows selected characteristics according to subject response category. Responders had significantly lower fold changes in susceptibility to d4T (median 1.0 vs. 1.6, $p = .003$) and lower baseline viral loads (median 4.26 vs. 4.74 $\log_{10}$ copies/mL, $p = .004$) than the nonresponders. Responders also had marginally significantly lower fold changes in susceptibility to ZDV (3.2 vs. 12, $p = .092$) and fewer TAMS at reverse transcriptase amino acid positions 41, 67, 70, 210, 215, and 219 (median 1 vs. 2, $p = .059$) than the nonresponders. d4T susceptibility was highly correlated with ZDV susceptibility (rho = .90, $p < .001$, Fig. 1A) and with the number of TAMS (rho = 0.84, $p < .001$, Fig. 1B). A weaker correlation was observed between d4T susceptibility and baseline HIV RNA level

TABLE 1. *Viral load reductions from baseline, D4T phenotype, and major reverse transcriptase mutations*

t	Change in HIV RNA from week 0 to 8 (log_{10} copies/mL)	d4T fold change 1	d4T fold change 2	Geometric mean D4T fold change	ZDV fold change	Reverse transcriptase changes from consensus B (TAMS in **bold**)
1	−0.95	1.0	NA	NAQ	2.0	V60I,**K70R,** V90I,S162A,T200A,E204D,R211Q,A272P,I293V
2	−0.86	1.0	1.0	1.0	3.4	**K70R,** D123E,I135V,T200I/V,R211K/R,R277K/R
3	−0.79	0.9	1.0	0.95	2.1	E6D,V60I,***K70R,***R83K,V90I,I135T,S162A,T200A,Q207E,V245E
4	−0.78	1.0	1.1	1.05	3.2	**K70R,**A272P
5	−0.63	1.1	1.1	1.1	5.6	R83K,I132I/T,L187F/L,**T215N/S/T/Y,**K223K/N
6	−0.62	1.0	1.0	1.0	5.2	K49K/R,**K70K/R,**R83R/K,D123D/E,I135T,S162C/Y,E169A/E,I202V,L228L/R, R277K
7	−0.52	0.9	1.0	0.95	1.6	V60I/V,**K70R,**R83K,D123E,D177E,I178L,V179I,T200A/T,R211K
8	−0.29	1.7	1.7	1.7	27	T39A,**M41L,**V60I/V,K122E,D123S,I135M,S162Y,V179I,**T215Y,**R284K,T286A, I293V,T296S/T,E297R
9	−0.26	1.0	NA	NA	2.2	K20R,K49R,I135T,S162C,V245T,Q278H/Q,I293V
0	−0.25	0.9	0.9	0.9	0.9	V35T,E36A,T39E,E40D,D123E,K173A,D177E,T200A,V245O,P247P/T,D250D/E, A272P,I293V,P294T
1	−0.25	1.6	NA	NA	25	V35I,**D67N,K70R,**D123E,I135T,N136I/N,I142V,D177E,R211E/K,**T215Y,K219Q,** H235H/R,V245M
2	−0.19	1.9	1.6	1.74	42	**M41L,**K43K/N,Q207E,R211K/R,**T215Y,**A272P
3	−0.15	1.7	2.0	1.84	79	**M41L,**I135T,S162C,**T215Y,**V245K,S251I,A272P
4	−0.13	1.6	2.2	1.88	101	K20R,V60I,**D67N,K70R,**K104N,T200A,I202V,Q207A/E,R211K/R,**T215F,K219Q,** T286A,I293V
5	−0.12	1.7	NA	NA		R20K,K64R,**K70R,**I135T,S162H,E204D/E,Q207D,R211K,**T215Y,**M230M/V, T286A,E297K,A299A/G
6	−0.11	1.1	1.1	1.1	4.3	K64K/R,**K70R,**K82K/R,A98S,K104R,Q174H,R211K,F214F/L,V245E/K/M/V. S251I,A272Q
7	0.01	1.4	1.7	1.54	3.9	**K70R,**S162C,**T215Y,**A272P,E297K
8	0.07	1.2	1.2	1.2	7.1	E36D,**K70R,**D121Y,K122E,I135L,S162C,D177D/E,V179I/M/V,V245M/T
9	0.15	4.1	4.2	4.15	>1345	K20R,V35M,K43D/E/K/N,E44D/E,**M41L,**V60I,**D67N,**K104N,K122E,**L210W,** R211K,**T215Y,**V245Q
0	0.16	1.7	1.7	1.7	12	T39A,S48T,**D67N,K70R,**K103R,K122E,D123N,I135T,S162I,Q197E,T200I,F214L, **K219Q,**L228H,A272P,R277K,I293V,E297A
1	0.21	1.2	NA	NA	5.5	N57H/N,**K70R,**F77F/L,A98A/S,I142V,Q197P/Q,T200A,Q207H/Q,**T215N/Y, K219K/M**
2	0.21	0.9	1.0	0.95	1.0	A98S,K122E,G196E,T200A,A272S,K281R,I293V
3	0.23	1.0	1.2	1.1	1.4	K20R,E248D/N
4	0.27	1.6	1.8	1.7	17	T39A,**D67N,K70R,**K103R,T200K,I202V,Q207E,R211K,F214L,**K219Q,**A272P
5	0.32	1.4	1.8	1.59	10	**K70R,**R83K,K122P,I142V,A158S,D177E,T200A,Q207E,**T215N/S/T/Y,**A272S, T286A,E297A
6	1.33	1.6	2.2	1.88	83	**D67N,K70R,**V90I/V,I135M,S162C,Q174H,G196E,Q207R,**K219Q,**V245K,A272P, K281K/R,T286A

NA, not available, d4T, stavudine; ZDV, zidovudine.

(rho = 0.40, $p = .045$; data not shown). The coefficient of variation for d4T susceptibility was 10%.

Among the variables evaluated, d4T fold change was the strongest predictor of the change in HIV-1 RNA at week 8 from baseline. In univariate regression analysis, where outcome was measured as the quantitative change in HIV RNA from baseline to week 8, each doubling in d4T fold change (e.g., from a fold change of 1 to 2, or from 2 to 4) was significantly associated with an additional 0.46 log_{10} copies/mL smaller reduction in HIV-1 RNA at week 8 from baseline (Table 3). For example, the regression model gave an estimated decrease in HIV-1 RNA of 0.35 log_{10} copies/mL for a subject with a d4T fold change of 1, compared with an increase of 0.11 log_{10} copies/mL for a subject with a d4T fold change of 2 (a difference in estimates of 0.46 log_{10} copies/mL). There were also marginally significant trends for poorer reductions in HIV-1 RNA between baseline and week 8 for higher ZDV fold change and higher number of TAMS in univariate analysis (Table 3). In multivariate regression analysis, decreased d4T susceptibility was the only significant predictor of poorer reductions in HIV-1 RNA between baseline and week 8 (Table 3).

TABLE 2. *Univariate analysis of baseline characteristics in nonresponders vs. responders expressed in medians with interquartile ranges (25%, 75%)*

	Nonresponders ($n = 19$)	Responders ($n = 7$)	p value
Age, years	39.5 (33.0, 43.1)	41.9 (31.9, 46.6)	.50
HIV-1 RNA, log_{10} copies/mL	4.74 (4.38, 5.21)	4.26 (3.89, 4.37)	.004
Stavudine fold change	1.6 (1.1, 1.8)	1.0 (1.0, 1.1)	.003
Zidovudine fold change	12 (2.2, 60)	3.2 (2.0, 5.2)	.092
Number of thymidine analog mutations	2 (1, 3)	1 (1, 1)	.059

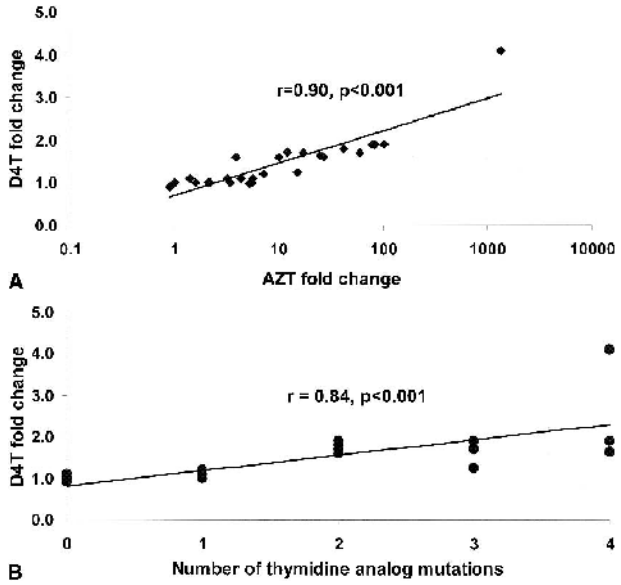

FIG. 1. **(A)** Relationship between zidovudine (ZDV) and stavudine (d4T) phenotype. **(B)** Relationship between d4T phenotype and number of thymidine analog mutations.

Based upon the initial phenotypic result for each sample, all 7 subjects who achieved a virologic response had d4T fold changes between 0.9 and 1.1. The positive predictive value (the proportion of patients with a value less than or equal to the threshold who had a virologic response) and negative predictive value (the proportion of patients with a value greater than the threshold who did not have a virologic response) of a 1.1-fold threshold for predicting virologic response was evaluated. In this study, the negative predictive value for a threshold of 1.1 was 100% when based upon a single d4T phenotypic assessment. Because there were 12 subjects with a single d4T fold change of less than or equal to 1.1, the positive predictive value of this threshold was 7/12 (58%). Comparably, the lowest ZDV fold change that gave a negative predictive value of 100% was 5.6; the positive predictive value for this cutoff was 47%.

Natural variation in d4T susceptibility exists in HIV-1 isolates from antiretroviral-naive patients ("biologic cutoff"). In an analysis of HIV-1 isolates from 240 antiretroviral-naive patients, 100% of the isolates had d4T fold changes in susceptibility 1.4 or less with the PhenoSense assay (Fig. 2) (ViroLogic, Inc., unpublished data). Using a d4T fold change of $\leq$1.4 as the cutoff, the negative predictive value remained 100%, and the positive predictive value decreased to 44% (Fig. 3).

DISCUSSION

This study demonstrated that even small changes in susceptibility to d4T (fold changes >1.1) in patients who had previously received only ZDV monotherapy were associated with a lack of short-term viral load reductions to d4T monotherapy. Baseline d4T phenotype was superior to baseline viral load, number of TAMS, and ZDV phenotype in predicting viral load reductions from baseline to 8 weeks in both univariate and multivariate analyses (Tables 2 and 3).

Previous investigations of the phenotypic assay used in this study have shown that viruses from antiretroviral-naive patients have a natural variability of d4T susceptibility, but all viruses have a d4T fold change 1.4 or less. At d4T fold change values of 1.4-fold, 44% of the patients in our study achieved viral load reductions of >0.3 $\log_{10}$ copies/mL from baseline, compared with none of the subjects with fold changes greater than 1.4. Although d4T phenotype was superior to ZDV phenotype at predicting response to d4T monotherapy, the two were highly correlated. The lowest ZDV phenotypic cutoff with a negative predictive value of 100% for response to d4T was 5.6, which gave a slightly lower positive predictive value (47%) than the d4T phenotypic cutoff of 1.1 (58%).

A possible reason for the relatively low positive predictive value of 44% in those subjects with a d4T phenotype of $\leq$1.4 is poor medication adherence. Four subjects with d4T fold changes of 0.9 or 1.0 did not respond to therapy and had no baseline TAMS (Table 1) despite a history of viral replication during prolonged ZDV monotherapy. This suggests inadequate exposure of the virus to ZDV and may reflect similar inadequate exposure to d4T during our study period.

The high correlation between d4T and ZDV phenotype likely explains the large changes in the regression parameter estimates for d4T and ZDV susceptibility between the univariate and multivariate regression models (Table 3), including the negative estimate (albeit nonsignificant) for ZDV susceptibility. This study is limited by its small sample size and hence these estimates are also imprecise, as evidenced by the wide confidence intervals (Table 3). Larger studies will be needed to elucidate further the potential contribution of ZDV phenotype in addition to d4T phenotype in predicting virologic response.

d4T phenotype was also positively correlated with the number of TAMS. Number of TAMS was, however, a weaker predictor of virologic outcome at week 8 in analyses comparing responders to nonresponders (Table 2) and quantitative changes in HIV RNA from baseline. This may reflect components of d4T resistance captured by the d4T phenotype over and above the number of TAMS, such as the relative importance of the individual

TABLE 3. *Regression analyses of the change in HIV-1 RNA at week 8 of D4T therapy from baseline*

	Univariate analysis[a]			Multivariate analysis[a]		
Baseline variable	Estimated increase in HIV-1 RNA at week 8	95% confidence interval	*P* value	Estimated increase in HIV-1 RNA at week 8	95% confidence interval	p value
d4T susceptibility (per 1 $\log_2$ FC increase*)	0.46	0.11, 0.82	.012	1.06	0.03, 2.10	.045
HIV-1 RNA (per 1 $\log_{10}$ copies/ml increase)	0.20	–0.19, 0.58	.30	0.00	–0.40, 0.39	.98
ZDV susceptibility (per 1 $\log_{10}$ FC increase*)	0.22	–0.03, 0.46	.079	–0.47	–1.13, 0.20	.16
Thymidine analog mutations (per 1 additional mutation)	0.15	0.00, 0.30	.054	0.04	–0.23, 0.30	.78

[a] In the univariate analysis, each baseline variable was considered in a separate regression model (so 4 models total). In the multivariate analysis, all baseline variables were included in the same mode.

[b] A 1 $\log_2$ FC increase corresponds to a doubling in FC, e.g., from 1 to 2, or from 2 to 4. A 1 $\log_{10}$ FC increase corresponds to a 10-fold increase in FC, e.g., from 1 to 10, or from 10 to 100.

FC, fold change; d4T, stavudine; ZDV, zidovudine.

TAMS, as well as the importance of other mutations. It may also reflect greater power to detect an association using a more quantitative measure of resistance, such as d4T phenotype, than a measure that takes a more limited set of values (subjects in this study had between 0 and 4 TAMS).

This study provides further evidence that there is a significant degree of cross-resistance between ZDV and d4T despite the limited degree of phenotypic expression of resistance observed to d4T in all the currently available assays. Various combinations of mutations at codons 41, 67, 70, 210, 215, and 219 have been shown to mediate ZDV and d4T resistance by facilitating ATP-dependent removal of a dideoxy-nucleotide monophosphate from a terminated DNA chain, which enables the chain to resume elongation (18,19). In a nucleotide-terminated primer, the presence of the next nucleotide (dNTP) that would have been incorporated into the chain had the primer been free for elongation results in the formation of a stable dead-end complex between the reverse transcriptase, primer, template, and dNTP. This complex interferes with the ATP-mediated chain rescue. ATP-mediated rescue of ZDV-terminated primers is more likely to occur than rescue of other nucleotide-terminated primers at the dNTP concentrations present in activated cells, perhaps because the bulky azido group of ZDV may interfere with the formation of the dead-end complex by sterically preventing the addition of the next dNTP. At diminishing levels of cellular dNTPs, which may occur in vivo, the rate of ATP-dependent rescue of d4T-terminated primers is enhanced (20). This could explain why ZDV expresses higher levels of in vitro phenotypic resistance compared with other nucleoside analogs like d4T in the presence of TAMS despite the

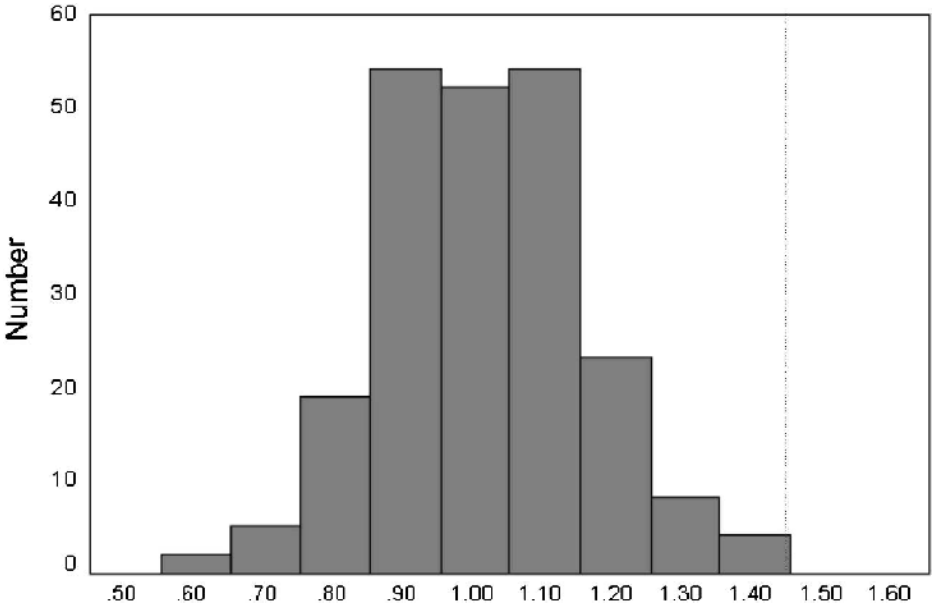

FIG. 2. Stavudine (d4T) phenotypes among 240 antiretroviral-naive subjects. One hundred percent of subjects were ≤1.4-fold change in susceptibility over the wild-type control using the PhenoSense assay (Hellman et al., unpublished data).

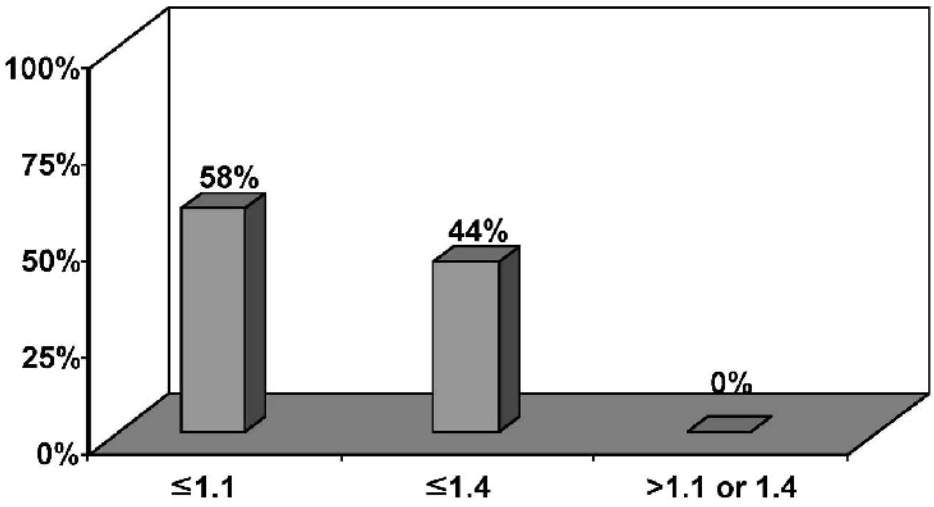

FIG. 3. Positive predictive values according to stavudine (d4T) phenotypes. Response rates based on the lowest d4T fold change that gave a negative predictive value of 100% (1.1) and the lowest fold change taking into consideration the biologic cutoff of d4T.

substantial clinical cross-resistance shown in our study and others (7,8).

In summary, very small changes in baseline d4T susceptibility (>1.1-fold change) predicted a poor short-term virologic response to d4T monotherapy in these ZDV-experienced patients, providing further clinical evidence that the thymidine analogs are highly cross-resistant. Further analyses with larger numbers of patients will be required to better delineate the best phenotypic cutoff of d4T.

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