Multiple sites in HIV-1 reverse transcriptase associated with virological response to combination therapy

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Objective: To determine whether analysis of sequence variation in reverse transcriptase at baseline can explain differences in response to combination antiretroviral therapy.

Methods: Amino acid sequences of reverse transcriptase obtained from baseline isolates from 55 patients included in a trial of zidovudine and didanosine versus zidovudine/didanosine/nevirapine (ACTG241) were analysed. Simple and multiple linear regression were used to determine the relationship between numbers and identity of mutations at baseline and virological response after 8 and 48 weeks.

Results: Numbers of baseline zidovudine resistance mutations were predictive of short-term response (week 8). Amino acid identity at position 215 explained > 20% of the variation in response at week 8, but less at week 48. Multiple regression identified the combinations: 215 + 44 and 41 + 202, each of which explained about 30% of the variation in week 8 response. A model incorporating amino acids 214 + 215 + 60 + 202 + baseline viral load explained > 40% of the variation in response at week 48. Unexpectedly, the mutant combination 60I + 215Y/F responded threefold better than 60V + 215Y/F over 48 weeks.

Conclusions: Use of clinical data to analyse virological response to combination therapy has revealed effects of baseline amino acid mutations at sites not previously identified as being important in antiretroviral resistance. Predictors of long-term responses were different from those involved in the short term and may require more complex analysis.

AIDS 2000, **14**:31–36

Keywords: antiretroviral therapy, HIV drug resistance, HIV sequence variability, viral load, reverse transcriptase inhibitors

Introduction

Mutations in the HIV-1 *pol* gene that contribute to drug resistance have been identified for all of the currently available antiretroviral agents by their frequent appearance under drug pressure in culture and in patients failing therapy. High level resistance may be conferred by a single amino acid substitution [1–4] or,

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Sponsorship: Supported by grants TW00767 (Fogarty Center), K 11 AI01361, AI 27670, AI 38858 AI 36214 (Center for AIDS Research), AI 29164, AI 32775, 96VC001, AI 40876, and AI 27767 (Center for AIDS Research) from the National Institutes of Health. Further support was provided by the Research Center for AIDS and HIV Infection of the San Diego Veterans Affairs Medical Center and the Core Laboratory Research Facilities of the University of Alabama at Birmingham School of Medicine, University of Alabama at Birmingham Center for AIDS Research and the Birmingham Veterans Affairs Medical Center. Heather Precious was supported by the Medical Research Council and Huldrych Günthard was supported by the Swiss National Science Foundation (No. 84 AD-046176).

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Received: 5 August 1999; revised: 8 October 1999; accepted: 14 October 1999.

particularly in the case of zidovudine (ZDV) and protease inhibitors, may require the stepwise accumulation of a number of mutations of incremental effect [5–7]. Treatment with more than one drug, sequentially or in combination, can lead to the development of multiply resistant strains [8,9]. As the emergence of drug resistant mutants has been shown to correlate with resurgence in plasma RNA levels [2,4,10], information on resistance mutations at the outset of therapy might predict virological, and ultimately clinical, response, as declines in viral load during antiretroviral therapy are associated with a decreased risk of disease progression [11–14]. It has recently been shown that short-term virological response to salvage therapy is better predicted by the number of mutations associated with resistance to antiretroviral agents present at baseline than by drug history, viral load or CD4+ cell count [15]; high level ZDV resistance, and ZDV resistance mutations at positions 215 and 41 have been found to be predictive of disease progression during treatment with ZDV or didanosine (ddI) [16–18].

The properties of specific mutations can be ascertained *in vitro* by the change in drug sensitivity conferred by the introduction of the mutation(s) to a defined genetic background. In this way, the additive and synergistic contributions of individual mutations to the development of resistance have been determined [1,3,5,6,19]. Antagonism between mutations, such as the suppression by M184V or Y181C of the ZDV resistance conferred by T215Y, has also been detected [1–3,19]. However, alternative resistance profiles arise in different patients which vary either because of the effects of pre-existing polymorphisms and resistance mutations [9,20–22] or as a result of random events in the evolution of the viral population [23,24]. As more patients are exposed to greater numbers of drugs, the complexity of the patterns of mutations seen in clinical isolates increases making it difficult to identify the best predictors of the outcome of combination therapies.

We have analysed the nucleotide sequences of HIV isolates obtained from patients enrolled in a randomized, double-blind, placebo-controlled trial of ZDV/ddI versus ZDV/ddI/nevirapine (ACTG241 [25]). A total of 398 ZDV-experienced patients with CD4+ cell counts $\leqslant 0.35 \times 10^6$/l completed the trial, and patients receiving the triple combination showed a $0.25 \log_{10}$ lower mean plasma viral load, and 18% higher mean CD4+ cell count, after 48 weeks than patients receiving dual therapy [25]. Sequences of reverse transcriptase (RT) were obtained from a selected subset of patients, at weeks 0, 8 and 48 (Hanna, Johnson, Kuritzkes, Richman, Leigh Brown, Savara, Hazelwood, D'Aquila, unpublished data). We have applied simple and multiple linear regression analysis to data available from 55 patients and have found that a substantial proportion of the variation in the ratio of the baseline viral load to viral load at week 8 and week 48 ('virological response') can be explained on the basis of sequence variation present at baseline. This approach has shown that whereas some sites identified from *in vitro* studies are important, others not previously known to be associated with antiretroviral resistance play a significant role in the clinical context.

Methods

Sequence analysis

A 1.2kb DNA fragment of HIV-1 *pol* was amplified by PCR from lysates of infected cultured cells and sequenced using high-density oligonucleotide arrays (Affymetrix, Santa Clara, California, USA), as described previously [26]. The baseline nucleotide sequences of the RT coding region (codons 1–242) from 55 patients were aligned using the Genetic Data Environment multiple sequence editor and all subsequent analyses were performed on inferred amino acid sequences of RT. Amino acids at each site were classified as mutant or non-mutant relative to the consensus for clade B [27]. Different mutant amino acids at the same site, such as 215Y and 215F, were combined.

Viral quantification

HIV plasma viral RNA was quantified with the Amplicor Monitor assay (Roche Molecular Systems, Branchburg, New Jersey, USA), with a cut-off of 200 copies/ml. Undetectable viral loads were given a value of 200 for calculation of virological response. Virological response was defined as the ratio of the baseline viral load to the viral load at week 8 or week 48. For statistical analysis these values were normalized by $\log_{10}$ transformation.

Statistics

Statistical analyses were performed using Microsoft Excel, SPSS for Windows version 6.1.3 and Minitab for Windows version 11.2. Multiple regression models were identified using stepwise and best subsets approaches. For stepwise regression, the criteria for entry and removal were set as $P_{in} = 0.05$ and $P_{out} = 0.10$. Best subsets regression identified combinations of variables with the highest value of R^2 for each number of parameters (1–20) in the model. As the number of free predictors in the model is limited to 20, it was necessary to repeat the best subsets procedure: starting with the 20 variables with the highest value of R^2 in bivariate regression, the last five (or six if including RNA) variables to enter any multiple regression model were repeatedly removed until all the models containing up to at least four (five) parameters (the maximum number for which all variables were likely to be significant, as suggested by stepwise regression) were constant. In order to compare models with different

numbers of parameters, R^2 values for multiple regression models were adjusted for the number of variables. The adjusted value, adj R^2, is equal to $R^2 - \{p(1 - R^2)/(n - p - 1)\}$, where p is the number of independent variables in the equation. The numbers in each treatment arm were too small to analyse separately, but there was no difference in mean virological response between arms in the patients used in this analysis.

Table 1. Number of mutations in baseline sequences and virological response to therapy in ACTG241.

Number of mutations from consensus	n	Mean	R^2	P
Total mutations				
Week 8	52	6.4	0.0809	0.041
Week 48	47	6.5	0.0058	0.612
ZDV associated				
Week 8	52	2.5	0.1917	0.0012
Week 48	47	2.5	0.0409	0.1729

Results

Bivariate regression

Sequences of the RT coding region between amino acids 1 and 241 were obtained from 55 patients. Substantial diversity was detected in the amino acid sequences obtained from baseline samples: 25 amino acid sites showed a variant in six or more patients. These included six sites associated with ZDV resistance (41, 67, 70, 210, 215 and 219), but none specifically associated with ddI or nevirapine resistance. At week 48, 33 sites were classified as variable, including 103, 106, 181 and 190 which are associated with resistance to nevirapine. For 52 patients for whom week 8 viral loads were available, and 47 patients with week 48 data, bivariate regression was performed of virological response at each time point on normalized baseline viral load ('baseline RNA'). Although baseline viral load was positively correlated with week 48 viral load, ($r = 0.90$, $P < 0.001$) the measure of virologiccl response adopted was not correlated with baseline RNA at either time-point.

The number of mutations present at baseline and the number of ZDV-associated mutations was analysed in relation to virological response. The total number of mutations present at week 0 in each patient at the 25 variable sites ranged from 1 to 15 (mean, 6.5). A greater number of mutations at baseline was significantly associated with a poorer response at week 8 ($R^2 = 0.08$, $P = 0.0410$). When only the number of mutations at the six sites associated with ZDV resistance [5,28] (mean, 2.5; range, 0–4) were considered, the significance was increased ($R^2 = 0.19$, $P = 0.0012$). However, numbers of mutations were not predictive of response at week 48 using either approach (Table 1).

Amino acid identity at baseline at five sites was found to have a significant individual effect on virological response at week 8 (Table 2). Three of these, 41, 210 and 215, are well known to have a major impact on susceptibility to ZDV. Two other sites which had significant individual effects were amino acid (aa) 44 and aa 39, where variant amino acids were observed in seven and six individuals respectively. In contrast, virological response at week 48 (Table 2) was affected by baseline amino acids at 214 and 215, and weakly by aa 196 and 200, thus only aa 215 is common to both sets.

Table 2. Bivariate regression for amino acid variants in week 0 sequences on virological response. The median value of the viral load ratio (ratio of baseline viral load to viral load following therapy) for patients bearing mutant and wild-type alleles at baseline is shown. A lower ratio indicates a poorer response.

Amino acid	R^2	P	Viral load ratio (mutant)	Viral load ratio (wild-type)
Week 8				
215	0.2105	0.0006	1.87	11.91
41	0.2103	0.0006	0.99	4.19
44	0.1868	0.0014	0.58	3.35
39	0.1029	0.0204	0.68	3.19
210	0.0836	0.0376	1.39	3.64
Week 48				
214	0.1544	0.0063	6.56	0.98
215	0.1470	0.0078	0.74	3.84
196	0.0980	0.0321	3.65	0.97
200	0.0937	0.0364	5.51	0.98

The median values for the viral load ratios for patients with mutant versus those with wild-type amino acids at baseline differ by two- to sixfold (Table 2). As expected, mutations at aa 215, 41 and 210 were associated with a poorer response to therapy (lower ratio), and patients with mutations at positions 44 and 39 also responded less well. Mutations in baseline sequences at aa 214, 196 and 200 were associated with a stronger response to therapy at week 48 (higher ratio, Table 2).

Multiple regression

Stepwise multiple regression identified aa 215 and 44 on the basis of virological response at week 8 (adj $R^2 = 0.291$, $P = 0.0001$). The same procedure selected a model involving four sites at week 48: aa 214, 215, 60 and 202 (adj $R^2 = 0.35$; $P = 0.0002$).

Best subsets regression was used to identify other combinations of sites which together explained the highest proportion of the variance in virological response. For response at week 8, this indicated that aa 41 and 202 together explained slightly more of the variation in response than did aa 215 and 44. Allowing three

parameters included aa 39 with 41 and 202, raising the adj R^2 from 0.311 (Table 3) to 0.347; the second greatest addition was with aa 44 (adj $R^2 = 0.342$ $P < 0.0001$). As the number of variables allowed in the model is increased, aa 215 and 200 (four or more variables), and baseline RNA (six or more variables) are included (Table 3). However, the increment in adj R^2 is small, to 0.37 with four variables and just 0.38 with five or more.

There was no significant difference in week 8 response between baseline 215Y and 215F ($P = 0.60$, n = 38). However, mutants at aa 41 always occurred in conjunction with 215Y (not 215F) (Hannah, Johnson, Kurtizkes *et al.*, unpublished data). Amongst the patients with the wild-type residue at aa 41 at baseline, those with 215F had a significantly worse response at week 8 than those with 215Y ($P = 0.05$, n = 14). Week 48 response did not differ significantly between 215F and 215Y, with or without allowance for aa 41 genotype ($P = 0.20$, n = 13; $P = 0.45$, n = 35 respectively).

For virological response at week 48, baseline RNA became significant in the presence of a mutation at aa 215, and significantly ($P = 0.002$) increased the value of R^2 of the four-site model (four sites and baseline RNA adj $R^2 = 0.478$; $P < 0.001$; Table 4), while also fullilling the stepwise criteria. Amino acids 200 and 214 were identified in the bivariate analysis, but aa 60 is significant only in a model which includes aa 215 as all 10 cases of 60I are found with 215Y/F. Sites 200 and 202 add a similar increment to R^2, in a four-site model but, unlike aa 200, 202 was significant only when 214 and 60 were included.

From the viral load ratios it appears that the presence of 60I substantially reduces the negative effects of 215Y/F. The ratio for aa 215 mutants overall is 0.74 whereas that for 60I 215Y/F is 1.65. The ratio for 60V, 215Y/F patients (n = 25) is 0.47, thus the presence of 60I has an approximately threefold effect on the viral load ratio after 48 weeks. Together with individuals who were wild-type at aa 215 at baseline, three groups can be identified with decreasing mean response at week 48: 60V 215T, 60I 215Y/F, 60V 215Y/F (Fig. 1). Mean baseline RNA is lower for the 215T class than for the 215Y/F classes, as described previously [29]. In the latter group, larger changes in viral load are associated with lower baseline values: patients with 60V 215Y/F make up the majority of those with low baseline RNA and poor response (Fig. 1).

In contrast with V60I, mutant amino acids at position 202 are associated with a poorer response. Thus, the four-site regression model indicates that variation at three amino acid sites which are not strongly associated with resistance can be combined with aa 215 in explaining the overall response on the trial. However, the inclusion of aa 60 and 202 in the model is unexpected on the basis of the relatively small effect on their own.

Associations between amino acid sites

The novel sites identified by the regression analysis (aa 60, 202, 196, 44 and 39) were tested for association with known resistance mutations and other variable sites. Significant associations were found between aa 60 and 215, referred to above (one-tailed $P = 0.035$, Fisher's exact test) and between aa 202 and both 219 and 41 (one-tailed $P = 0.01$ and 0.03, respectively). Although mutations at aa 41 and 219 are important for ZDV resistance, neither increased the R^2 value of the

Table 3. Multiple regression of amino acid variation in baseline sequences on virological response at week 8. The model with the highest value of adj R^2 is shown for each number of amino acid sites included, except for the model identified by stepwise regression indicated by *.

Number of variables	Amino acids	Adj R^2	*P*
2	215 + 44	0.291	0.0001*
2	41 + 202	0.311	< 0.0001
3	39 + 41 + 202	0.347	< 0.0001
4	215 + 39 + 41 + 202	0.365	< 0.0001
5	200 + 215 + 39 + 41 + 202	0.375	0.0001
6	208 + 215 + 44 + 41 + 202 + RNA	0.384	0.0001

Table 4. Multiple regression of amino acid variation in baseline sequences on virological response at week 48. The model with the highest values of adj R^2 is shown for each number of amino acid sites included. Model identified by stepwise regression is indicated by *.

Number of variables	Amino acids	Adj R^2	*P*
2	215 + RNA	0.258	0.0005
3	60 + 215 + RNA	0.380	< 0.0001
4	60 + 202 + 215 + RNA	0.422	< 0.0001
5	60 + 202 + 214 + 215 + RNA	0.478	< 0.0001*
6	69 + 60 + 200 + 214 + 215 + RNA	0.510	<0.0001

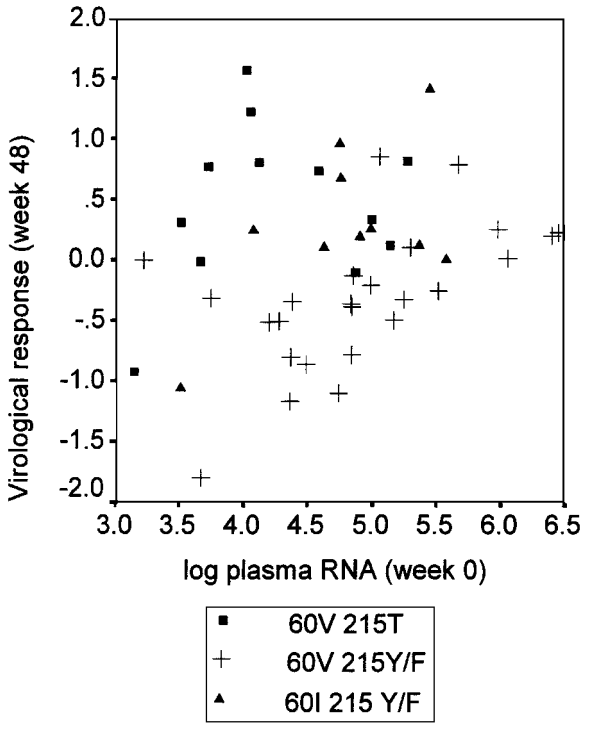

Fig. 1. Virological response at week 48 versus baseline RNA for baseline genotypes at aa 60 and 215. A response < 0 indicates an increase in viraa load from baseline to week 48.

model containing aa 215, 214, 60 and baseline RNA significantly, whereas inclusion of aa 202 did. Thus the effect of aa 202 on virological response is not due to its association with these two sites. Amino acid 60 showed no association ($P > 0.05$) with sites other than 215 in baseline sequences, but did show associations with mutations at aa 39 and 211 in week 48 sequences (one-tailed $P = 0.038$ and 0.046 respectively, data not shown).

Discussion

While the likely outcome of ZDV monotherapy can be predicted on the basis of the presence or absence of mutations at aa 215 and 41 [5], in combination therapy it is not yet clear what information is required to predict a response. We have analysed whether baseline sequences can be informative in predicting outcome to therapy, using two approaches. We have tested the predictive capacity of genotype at individual aa sites, including 215, by a simple bivariate regression, and secondly, we have applied multiple regression methods to the variation in the baseline sequences. Lorenzi *et al.* found baseline numbers of RT and protease inhibitor resistance mutations to be predictive of virological response following 4–12 weeks of salvage therapy including nelfinavir [15]. We have found that, although numbers of ZDV resistance mutations are predictive of response after 8 weeks, they do not predict long-term response to combination therapy, for which the baseline amino acid identity at key sites is more important. Numbers of mutations might, however, retain their predictive capacity during long-term therapy with protease inhibitors, resistance to which is characterized by overlapping profiles of multiple mutations.

Simple regression of individual amino acid sites identified five with a significant effect at week 8, two of which, aa 44 and 39, have not previously been shown to be strongly associated with antiretroviral resistance. It has recently been reported that 44D may arise during treatment with ZDV and, in a ZDV resistance-associated background, is associated with intermediate levels of resistance to lamivudine [30]. Furthermore, a study of patterns of resistance mutations selected during ACTG241 suggests that 44D is selected by nucleosides, on a background of 41L and 215Y (Hanna, Johnson, Kuritzkes Richmond, Leigh Brown, Savara, Hazlewood, D'Aquila, unpublished data). Different sites were identified when virological response at week 48 was analysed. While these included aa 215, positions 214, 196, and 200 were also found to be significant.

Multiple regression gave contrasting results for week 8, and week 48. While a two-parameter model of $215 + 44$ or $41 + 202$ explained approximately 30% of the response at week 8, significantly better performance at week 48 was obtained from a model including aa 214, 215, 60, 202 with baseline viral load. Clearly, although 215 retains an important influence on virological outcome in the long term, other less well understood sites are also important.

It is particularly interesting to consider the interaction between position 60 and 215 in this case as aa 60 has not been identified previously as being associated with either nucleoside or non-nucleoside RT inhibitor resistance. From these results it is clear that despite having a small effect when considered on its own, it has a substantial influence on outcome in the presence of 215Y/F, reducing the negative effect of mutants at 215 on the response to long-term therapy. Residue 60I is a common polymorphism in some subtypes [27] and has been recorded by Najera *et al.* in one out of 25 antiretroviral-naive patients, where it occurred with wild-type 215T, and in one out of 35 nucleoside-experienced patients (with 215Y) [31]. It has also been seen to arise on a 215Y background during prolonged ZDV therapy, but this was not accompanied by a quantifiable change in *in vitro* resistance to ZDV [33]. The appearance of 60I solely in conjunction with 215 mutants in ACTG241 patients suggests that it confers some immediate fitness advantage in the presence of nucleoside inhibitors, but that in the long term it clearly reduces the virus' ability to respond to continuing therapy.

In view of the increasing ease of obtaining genotypic data from HIV sequences, the ability to predict responses to antiretroviral therapy on the basis of information on sequence variation at baseline is of direct clinical relevance. We have shown that analysis of clinical data using multiple regression can identify associations of specific amino acid sites with response to combination therapy. In an analysis based on viral isolates we have found some sites to be important which have not been identified *in vitro* as having a role in antiretroviral resistance. It is likely that a similar analysis of sequences obtained directly from patients' plasma would be even more powerful, because of the possibility of loss of viral variants during culture in the absence of drug. We have also shown that the prediction of short-term and longer term responses may involve different parameters. While this analysis does not allow any direct inference regarding mechanisms, all of these conclusions can be tested directly by investigation of independent datasets.

References

1. Richman D, Shih CK, Lowys I, *et al.* **Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture.** *Proc Natl Acad Sci USA* 1991, **88**:11241–11245.
2. Richman DD, Havlir D, Corbeil J, *et al.* **Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy.** *J Virol* 1994, **68**:1660–1666.
3. Tisdale M, Kemp, SD, Parry NR, Larder BA. **Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase.** *Proc Natl Acad Sci USA* 1993, **90**:5653–5656.
4. Schuurman R, Nijhuis M, van Leeuwen R, *et al.* **Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC).** *J Infect Dis* 1995, **171**:1411–1419.
5. Kellam P, Boucher CAB, Tijnagel JMGH, Larder BA. **Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance.** *J Gen Virol* 1994, **75**:341–351.
6. Molla A, Korneyeva M, Gao Q, *et al.* **Ordered accumulation of mutations in HIV protease confers resistance to ritonavir.** *Nature Med* 1996, **2**:760–766.
7. Condra JH, Holder DJ, Schleif WA, *et al.* **Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor.** *J Virol* 1996, **70**:8270–8276.
8. Larder BA, Kellam P, Kemp SD. **Convergent combination therapy can select viable multidrug resistant HIV-1 *in vitro.*** *Nature* 1993, **365**:451–453.
9. Shiraska T, Kavlick MF, Ueno T, *et al.* **Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides.** *Proc Natl Acad Sci USA* 1995, **92**:2398–2402.
10. Eastman PS, Mittler J, Kelso R, *et al.* **Genotypic changes in human immunodeficiency virus type 1 associated with loss of suppression of plasma viral RNA levels in subjects treated with ritonavir (norvir) monotherapy.** *J Virol* 1998, **72**:5154–5164.
11. Coombs RW, Welles SL, Hooper C, *et al.* **Association of plasma human immunodeficiency virus type 1 RNA level with risk of clinical progression in patients with advanced infection.** *J Infect Dis* 1996, **174**:704–712.
12. O'Brien WA, Hartigan PM, Martin D, *et al.* **Changes in plasma HIV-1 RNA and CD4+ lymphocyte counts and the risk of progression to AIDS.** *N Engl J Med* 1996, **334**:426–431.
13. Yerly S, Kaiser L, Mermillod B, Baumberger C, Hirschel B, Perrin L. **Response of HIV RNA to didanosine as a predictive marker of survival.** *AIDS* 1995 **9**:159–163.
14. Hughes MD, Johnson VA, Hirsch MS, *et al.* **Monitoring plasma HIV-1 RNA levels in addition to CD4+ lymphocyte count improves assessment of antiretroviral therapeutic response.** *Ann Intern Med* 1997, **126**:929–938.
15. Lorenzi P, Opravil, M, Hirschel B, *et al.* **Impact of drug resistance mutations on virologic response to salvage therapy.** *AIDS* 1999, **13**: F17–F21.
16. D'Aquila RT, Johnson VA, Welles SL, *et al.* **Zidovudine resistance and HIV-1 disease progression during antiretroviral therapy.** *Ann Intern Med* 1995, **122**:401–408.
17. St. Clair MH, Hartigan PH, Andrews JC, Vavro CL, Simberkoff MS, Hamilton JD. **Zidovudine resistance, syncytium-inducing phenotype, and HIV disease progression in a case-control study.** *J Acquir Immune Defic Syndr* 1993, **6**:891–897.
18. Japour AJ, Welles S, D'Aquila RT, *et al.* **Prevalence and clinical significance of zidovudine resistance mutations in human immunodeficiency virus isolated from patients after long-term zidovudine treatment.** *J Infect Dis* 1995, **171**:1172–1179.
19. Larder BA, Kemp SD, Harrigan R. **Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy.** *Science* 1995, **269**:696–699.
20. Rose RE, Gong YF, Greytok JA, *et al.* **Human immunodeficiency virus type 1 viral background plays a major role in development of resistance to protease inhibitors.** *Proc Natl Acad Sci USA* 1996, **93**:1648–1653.
21. Gomez-Cano M, Rubio A, Puig T, *et al.* **Prevalence of genotypic resistance to nucleoside analogues in antiretroviral-naive and antiretroviral-experienced HIV-infected patients in Spain.** *AIDS* 1998, **12**:1015–1020.
22. Kemp SD, Shi C, Bloor S, Harrigan PR, Mellors JW, Larder BA. **A novel polymorphism at codon 333 of human immunodeficiency virus type 1 can facilitate dual resistance to zidovudine and L-2′,3′-dideoxy-3′-thiacytidine.** *J Virol* 1998, **72**:5093–5098.
23. Leigh Brown AJ. **Analysis of HIV-1 *env* gene sequences reveals evidence for a low effective number in the viral population.** *Proc Natl Acad Sci USA* 1997, **94**:1862–1865.
24. Leigh Brown AJ, Richman DD. **HIV-1: Gambling on the evolution of drug resistance?** *Nature Med* 1997, **3**:268–271.
25. D'Aquila RT, Hughes MD, Johnson VA, *et al.* **Nevirapine, zidovudine, and dianosine compared with zidovudine and didanosine in patients with HIV-1 infection.** *Ann Intern Med* 1996, **124**:1019–1030.
26. Gunthard HF, Leigh Brown AJ, D'Aquila RT, *et al.* **Higher selection pressure from antiretroviral drugs in vivo results in increased evolutionary distance in HIV-1 pol.** *Virology* 1999, **259**:154–165.
27. Human Retroviruses and AIDS 1997: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. (Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory).
28. Schinazi RF, Larder BA, Mellors JW. **Mutations in retroviral genes associated with drug resistance.** *Int Antiviral News* 1996, **4**: 95–107.
29. Leigh Brown AJ, Günthard HF, Wong JK, D'Aquila RT, Johnson VA, Kuritzkes DR, Richman DD. **Sequence clusters in human immunodeficiency virus type 1 reverse transcriptases are associated with subsequent virological response to antiretroviral therapy.** *J Infect Dis* 1999, **180**: 1043–1049.
30. Bloor S, Hertog K, De Vroey V, Miller V, Sturmer M, Larder B. **Lamivudine – resistant HIV-1 clinical isolates lacking the Met184Val mutation have novel polymorphisms in RT.** *Third International Workshop on Drug Resistance and Treatment Strategies.* San Diego. June 1999 [abstract 25].
31. Najera I, Holguin A, Quinones-Mateu ME, *et al.* ***pol* gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy.** *J Virol* 1995, **69**:23–31.
32. Rubsamen-Waigmann H, Schroder B, Biesert L, *et al.* **Markers for HIV-disease progression in untreated patients and patients receiving AZT: Evaluation of viral activity, AZT resistance, serum cholesterol, b2-microglobulin, CD4+ cell counts, and HIV antigen.** *Infection* 1991, **19** (suppl 2): S77–S82.