Review

Association of antiretroviral resistance genotypes with response to therapy – comparison of three models

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Genotype-based resistance assays are commonly used to aid treatment in HIV-infected individuals failing antiretroviral therapy. The relationship between genotype and antiretroviral therapy comes mostly from in vitro assays of the response to a single drugs although there is a need for a prediction of clinical response to combination therapy. We have compared three different methods of analysing genotype data as a predictor of clinical response in a small clinical cohort of highly antiretroviral-experienced individuals failing therapy. No method performed well beyond 8 weeks into a new therapeutic regime. A model based on the number of 'primary' mutations was statistically significant, but a multiple regression model, which identified specific mutations explained threefold more variation in response. Optimal prediction in this dataset was given by a model obtained from a classification tree analysis, in which genotype at amino acid sites 135 and 202 were combined with amino acid site 184, which explained over 50% of the deviance in the data and had a classification success of 86%.

Introduction

Assessment of virological resistance to antiretroviral drugs has become widespread in the clinical management of HIV infection. Guidelines have been published on when a resistance assay is indicated [1]. While details vary, interpretation of both phenotypic and genotypic resistance assays is based on *in vitro* susceptibility of a viral strain to individual drugs. However, they are used to attempt to predict clinical outcomes of therapy involving combinations of three or four drugs, frequently targeting both reverse transcriptase (RT) and protease (PR).

There have been a number of studies of the role of antiretroviral resistance in clinical responses to antiretroviral therapy [2,3] *in vitro* susceptibility tests. DeGruttola *et al*. [4] analysed multiple datasets using a common methodology: specified mutations associated with each drug in the regimen were identified and a 'genotype sensitivity score' determined on the basis of the number of drugs for which evidence of resistance was present; virological response was analysed in terms of this. This analysis was predicated on the additional assumption that presence of any of the individual mutations confers clinically significant resistance to the maximum extent possible. Others [2,5] these studies showed that genotypic resistance was in some way related to clinical response, but they did not attempt to optimise the interpretation of the genotype data.

In previous work analysing data from a clinical trial of nucleoside-experienced patients, ACTG 241, we [3] analysed all variable amino acid sites in RT and in a multivariate analysis, we found that, of known AZT-associated mutations only T215Y/F was associated with virological response, other known sites not contributing independently of 215. In addition, we found that the mutation E44D, not previously associated with resistance, independently explained a significant proportion of the variation in response at week 8. This site was subsequently shown

in vitro to contribute to nucleoside resistance [6]. Thus, there is reason to believe alternative models could improve the prediction of clinical response from genotype data. In this study we have compared the performance on data from a small clinical cohort of patients failing combination therapy, of a model based on primary resistance-associated sites with one incorporating all variable amino acid sites, and with an analysis based on a Classification And Regression Tree (CART) approach based on all variable amino acids. We found that the latter substantially outperforms the first two.

Methods

Patients

This was a retrospective study of patients failing antiretroviral (ARV) therapy. Patients were recruited for this study if they were (1) undergoing combination antiretroviral therapy involving three or more drugs; and (2) failing this therapy on the basis of having had a plasma viral load (pVL) for at least two sequential viral load assays above 10 000 (70% of patients) or an initial viral load of 1000, which increased during the study period. Baseline was defined as the time of change in ARV therapy.

Table 1. Demographic and baseline laboratory data {AUTHOR: MALE AND FEMALE PERCENTAGES ADD UP TO 98%, PLEASE CHECK IF OK}

Demographics	
Median age (range)	38 years (25–63)
Sex, %	
Male	65
Female	33
Risk group, %	
MSM	22
Heterosexual	18
IVDU	27
Unknown	33
Baseline laboratory data	
Median log10 plasma RNA (range)	4.64 (2.86–6.56)
Median CD4 count (range)	112 (0–672)
ARV therapy	
Previous NRTI exposure, % (*n*)	100 (49)
Previous NNRTI exposure, % (*n*)	61 (30)
Previous PI exposure, % (*n*)	74 (36)
Median time from first therapy	3.5 years (0.25–9)
Median time from first NRTI therapy	3.5 years (0.25–9)
Median time from first NNRTI therapy	1.25 years (0.08–3.42)
Median time from first PI therapy	1.83 years (0.08–3.17)

*Demographic data available on 49 of 76 patients.
{AUTHOR: PLEASE DEFINE MSM AND IVDU IN FULL}ARV, antiretroviral therapy; NRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor; PI, protease inhibitor.

Patient demographic characteristics are presented in Table 1. In brief, all patients were Caucasian, and were attending either the Genito-Urinary Medicine outpatient clinic at the Royal Infirmary, Edinburgh, UK, or the Infectious Diseases Department at the Western General Hospital, Edinburgh, UK. Patients were 65% male. Among all patients, risk group distribution was: 22% MSM{AUTHOR: DEFINE IN FULL}; 18% heterosexual contact (male and female), 27% injecting drug use, 33% unknown. Median age: 38 years (range 25–63); median $\log_{10}$ baseline viral load: 4.6 copies/ml (2.9–6.6); median CD4 count (cells/ml) 112 (0–670). All patients had received nucleoside reverse transcriptase inhibitors (NRTI), 40% had received non-nucleoside reverse transcriptase inhibitors (NNRTI) and 80% protease inhibitors (PI), with a median of 3.5 years of ARV therapy (0.25–9).

RT-PCR. Peripheral blood samples were separated into plasma (stored at –70°C) and leukocytes (stored in liquid N_2, vapour phase). Plasma viral loads were obtained using the Roche Amplicor HIV-1 Monitor. Plasma RNA was extracted using the QIAamp Viral RNA extraction protocol (QIAGEN Ltd, UK). Extracted RNA (5µl) was reverse transcribed into viral cDNA via specific viral primer-initiated cDNA synthesis, using the Expand™ Reverse Transcriptase protocol (Boehringer Mannheim). cDNA was aliquoted in two vials, each containing 10 µl, and stored at –20°C. PCR was performed on the cDNA to produce a ~1.3 kb *pol* fragment (encompassing all PR and codons 1–358 of RT). Subsequent nested PCR was performed to obtain a PR fragment (297 bp), and a RT fragment (from codons 26 to 244, 654 bp).

Sequence analysis. Genotypic analysis was performed on baseline samples by automated population-based DNA sequencing, using dRhodamine or Big Dye™ terminator chemistry on an ABI 373A XL machine (Perkin Elmer, Foster City, Calif., USA). Sequences were aligned using the Genetic Data Environment multiple sequence editor [7]. The nucleotide sequences were translated and amino acids scored as wild-type (WT) or mutant (MT), relative to the consensus for clade B (mixtures of WT/MT were coded as MT because of their potential for rapid evolution to MT). It should be noted that this was a retrospective study: genotype information was not available to the clinicians at the time the new therapy was chosen.

Statistical analysis. Patients were included in the statistical analysis if, in addition to the criteria listed above, they had remained on a stable regime of combination ARV therapy for at least 8 weeks The outcome variable for analysis of response to therapy by linear regression

was the ratio of the pVL at baseline, defined as the initiation of a new therapeutic regime, to the pVL at 8, 12 and/or 24 weeks (values were normalized by $\log_{10}$ transformation). For logistic regression analyses an empirically defined outcome variable ('responder') was adopted, based on the observed distribution of viral load ratios, which was aimed at maximizing the power of the analyses. A ratio greater than 4 (for analyses of amino acid sites in RT) or 3 (for amino acid sites in PR) was classed as 'responder'. The criteria adopted for stepwise regression were: P_{in}=0.05, P_{out}=0.10. All R^2 values shown are adjusted for the number of parameters in the model. The Bonferroni correction was applied to probability values for multiple tests. These analyses were performed using SPSS version 10.0.5. Regression tree analyses were performed in S-Plus 2000 (MathSoft Inc. Seattle, Wash., USA) on the categorical response variable defined above and pruned to the minimum number of nodes that explained at least 50% of the deviance in response.

Results

HIV genotype data – phylogenetic analysis

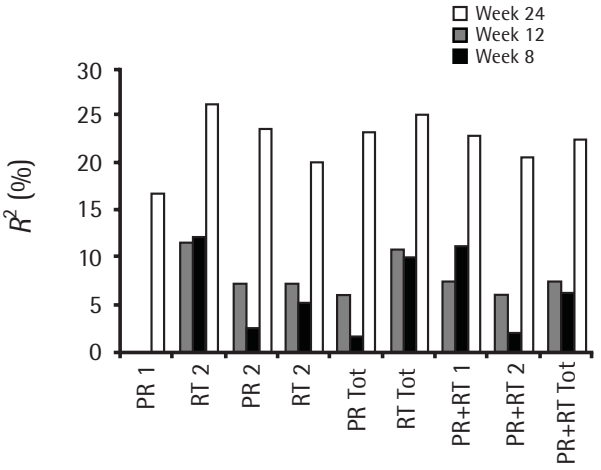
An alternative amino acid was observed in at least 5% of sequences at 29 (29%) of the 99 amino acid positions in PR and 54 (25%) of the 218 amino acid positions analysed in RT. As a check for sample contamination at any stage in the proceedings [8], a neighbour-joining phylogenetic analysis was performed on all sequences obtained from each gene. Only sequences shown to be distinct from all others by this test were included in subsequent analyses.

Sequence variation

Of eight 'primary' resistance-associated sites in RT [9], (amino acids 41, 103, 106, 151, 181, 184, 190 and 215), mutations were present at six among these patients (range over patients: 0–5); nine of 18 'secondary' sites (aa 62, 65, 67, 69, 70, 74, 75, 77, 98, 100, 101, 108, 115, 116, 179, 188, 210, 219) in RT were variable (range 0–7). For PR, out of 'primary' sites (aa 30, 46, 48, 82 and 90) mutations were observed at three (range 0–2) and at nine out of 12 'secondary' sites (aa 10, 20, 24, 32, 33, 36, 54, 63, 71, 73, 77, 84; range 1–7). Mutations associated with resistance to any NRTI were observed in 53/76 patients (70%); to any NNRTI in 36/76 (47%) and to any PI in 63/76 (83%). There was clearly substantial variation at sites associated with antiretroviral resistance among these patients, establishing that resistance is a significant issue for HIV therapy in this population.

Factors associated with response to therapy

Figure 1. Proportion of variance in response to therapy explained by models based on numbers of mutations associated with resistance



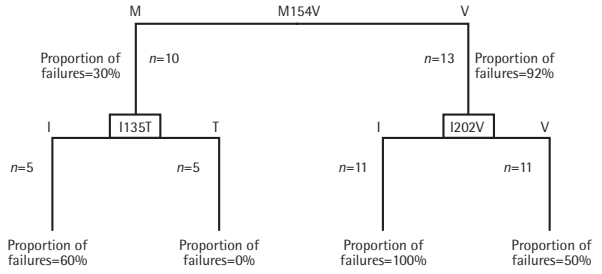
Proportion of variance explained by models incorporating primary and secondary mutations as defined in [9], for reverse transcriptase (RT) and protease (PR) alone and together. Code for analysed variables: 1=number of primary mutations; 2=number of secondary mutations; Total=1+2.

Number of resistance-associated mutations. Patients who remained on the same therapeutic regime until a pVL measurement was obtained at any of the three follow-up time points (8, 12 and 24 weeks from date of therapy change) were included in the analysis of response to therapy. We included in this analysis those individuals for whom complete (PR+RT) genotype and pVL data were available. For week 8, n=23, a subset of the datasets for weeks 12 and 24, where n=28.

Linear regression of number of mutations on virological response revealed major differences in the proportion of variation explained (R^2; Figure 1). Only for week 8 was 20% or more of the variation in response explained and only four associations were significant at P<0.01: RT primary, PR secondary, PR total (=primary + secondary) and RT total. Inclusion of PR with RT did not significantly increase the amount of variation explained. Baseline viral load has in previous studies been identified as acting independently of genotype. In this case it was not found to be correlated significantly with response when analysed on its own, nor was it included in a stepwise multiple regression model. Forcing it into the model increased the variation explained (adjusted {AUTHOR: CORRECT?} R^2 35% compared with 26% for primary RT mutations alone) but the change in significance was slight (P<0.005 vs P<0.007).

Specific mutations. It is known that for several antiretroviral drugs, mutations at only one or two positions are sufficient to impart resistance. These

Figure 2. Classification tree on amino acid sites in reverse transcriptase for response to therapy at week 8



Classification tree was obtained using the categorical variable 'Response' as defined in the text (>fourfold reduction in viral load). The tree was pruned up to increase the group sizes at the tips while still explaining 50% of the deviance. The amino acid sites, which define the nodes and the numbers of patients within each group, are shown next to the tree, together with the proportion of failures.

effects could be missed in an analysis based solely on number of resistance-associated mutations. We therefore performed bivariate regression taking each variable amino acid position as the independent variable to identify those sites which contributed most to the variation in response at each of the three time points (RT: Table 2; PR: Table 3). Three sites in RT, 184, 196 and 67 had *P* values lower than 0.01, and a further seven of 0.05 or below, but only 184, which on its own explained over 40% of the variation in response ($P<0.0003$), remained significant after correction for multiple tests. The proportion of variation explained by individual sites at other time points was always lower and none remained significant after correction. For PR, no site remained significant after correction at any time point.

To construct a model based on specific amino acid sites, all sites identified individually in Tables 2 and 3 were entered into stepwise multiple regression analyses. Significant models were obtained for weeks 8 and 12 based on sites in both RT and PR taken separately, and for all three timepoints when both genes were taken together (Table 4), although the P value for the week 24 model was only 0.011. For week 8, a model based on RT alone (aa 184+aa 196+aa211) was identified, which explained over 70% of the variation in response. When tested with PR, aa 211 was substituted by aa 12 in PR, but with no improvement in the significance, or the proportion of variation explained. For the week 12 timepoint the best performing model included two amino acids from PR, aa 12 and aa 63; a second model based on both genes had a lower R^2.

The predictive value of models based on (a) number of resistance-associated sites, and (b) on specific amino acid sites, can be compared on the basis of the proportion of variation in response explained by each (Figure 1 & Table 4). For week 8 this comparison shows the RT site-based model (type b) explaining nearly three times as much variation as the best type (a) model. We have also made this comparison using logistic regression on the week 8 RT dataset, defining responders as those cases where the ratio of baseline to week 8 viral loads was fourfold or higher and including baseline viral load. The odds ratio per primary resistance-associated mutation for model (a) was 0.5 (95% CI: 0.283–0.959), independent of baseline viral load. In the best model of type (b), the odds ratio for the presence of a mutation at amino acid 184 was 24 (95% CI: 2–290), taking account of the effect of baseline viral load, thus in this dataset 184 on its own was more important than other 'primary' mutations in determining response.

CART analysis

A third analytical method used to describe the association of amino acid variation and response was the classification/regression tree approach [10]. All variable amino acid sites in RT were included in this analysis, which was performed on the categorical response variable (viral load ratio >4=responder). Once again the best supported model was obtained on week 8 data for RT. In view of the small number of individuals to be partitioned, the tree was pruned to just three nodes, but still explained over 50% of the deviance in response. The classification tree model was concordant with the stepwise regression models in that M184V identified the first node (Figure 2), but added additional information by including aa 202 as identifying a second split in the 184V group and aa 135 as a second split in the 184M group. The three-site model correctly allocated 20/23 individuals (success rate: 86%). Among patients with valine at position 184, 11/13 were failures while 3/10 cases with 184M were failures. Among the latter, adding information on aa 135 improved precision: 0/5 cases with 184M+135T were failures, while in the other branch, 3/5 (60%) patients with 184M+135I were failures. However, most (9/14, 64%) patients with 135I were also 184V, in which background variation at aa 135 does not appear to contribute any significant effect. Thus while not contributing to the classification among the 184V group, aa 135 provided additional information about response of individuals with 184M. A parallel regression tree analysis, in which the dependent variable is continuous instead of categorical, again identified M184V as the split explaining the largest proportion of the deviance (data not shown).

Discussion

The aim of this investigation was to determine whether it was possible to improve the predictive power of

Table 2. Correlation of mutations at individual amino acid sites in RT with response to therapy

Amino acid	Week 8		Amino acid	Week 12		Amino acid	Week 24	
(WT %)	R^2	*P*	(WT %)	R^2	*P*	(WT %)	R^2	*P*
184 (44)	43.5	<0.001	178 (88)	23.2	0.009	184 (37)	15.7	0.023
196 (91)	35.3	0.003	118 (56)	14.3	0.029	98 (78)	12.5	0.04
67 (52)	27.4	0.006	207 (80)	15.1	0.03	123 (48)	10.6	0.05
41 (48)	20.3	0.018	41 (30)	13.2	0.035	215 (33)	11.0	0.05
215 (48)	20.3	0.018	67 (56)	11.7	0.045			
211 (23)	18.7	0.025	74 (85)	11.4	0.048			
178 (81)	19.2	0.027	184 (26)	10.0	0.06			
122 (44)	15.7	0.035						
123 (48)	14.1	0.043						
210 (52)	13.1	0.050						

WT, wild-type.

resistance genotype data. Previous analyses have shown significant associations between the presence at baseline of mutations associated with reduced susceptibility *in vitro* and clinical response [2,4,5,11]. Some of these studies used analyses which showed that the number of such mutations present was positively correlated with response. However, we previously showed in an analysis of an early clinical trial of combination therapy [3], that of the five mutations in RT known to be associated with zidovudine susceptibility, only 215Y was independently correlated with virological response. Thus, although addition of 41L to 215Y confers a substantial reduction in susceptibility *in vitro*, it did not add any predictive value to the model for the patient data. In fact, an unrelated mutation, 44D, not known at the time to be associated with antiretroviral resistance, was identified as significantly associated with response independent of 215. Subsequently, Hertogs *et al*. [12] revealed *in vitro* effects of this mutation on nucleoside analogue susceptibility, confirming the conclusions of the analysis of clinical response.

In the present study, we have attempted to find an optimal model for the prediction of response to therapy in a clinical cohort. In this group, number of primary resistance-associated mutations was significantly associated with response at week 8, explaining about 25% of the variance in response. Testing amino acid sites individually on this dataset showed that one site remained significantly associated with response after correction for repeated tests; this was aa 184 in RT, with an odds ratio of 24. On its own, this site explained over 40% of the variance in response, thus outperforming number of primary mutations. The optimal model for week 8 included the amino acid sites 196+211 with 184 and explained over 70% of the variation in response. At first sight it is surprising that no other 'primary' resistance-associated site was independently associated with response, which could be due to the small size of the dataset analysed, but it may suggest that the high level of association among these sites means they do not, therefore, provide independent information, as first seen in the analaysis of ACTG 241 [3]. Amino acid 184 is clearly a strong candidate for a key mutation in this dataset as over 80% of the study patients were receiving lamivudine in their current

Table 3. Correlation of mutations at individual amino acid sites in protease with response to therapy

Amino acid	Week 8		Amino acid	Week 12		Amino acid	Week 24	
(WT %)	R^2	*P*	(WT %)	R^2	*P*	(WT %)	R^2	*P*
64 (83)	32.0	0.003	12 (89)	31.9	0.001	64 (71)	17.2	0.018
35 (61)	30.5	0.004	13 (78)	24.7	0.005	20 (78)	13.0	0.036
12 (86)	19.2	0.024	64 (78)	25.1	0.005	35 (63)	10.5	0.055
71 (61)	18.2	0.024	63 (85)	18.3	0.015			
20 (70)	18.1	0.025	72 (85)	17.6	0.017			
90 (61)	16.7	0.03	54 (74)	15.4	0.025			
37 (70)	15.2	0.037						
41 (70)	13.5	0.047						
54 (74)	13.1	0.05						

WT, wild-type

Table 4. Multiple regression models based on mutations at specific amino acid sites in protease and reverse transcriptase

Dataset	Amino acid sites	*R*	SE (*R*)	Adj. R^2	*P*
Week 8 RT	184+196+211	0.796	0.64	0.719	0.0001
Week 8 PR	64+35	0.71	0.73	0.452	0.001
Week 8 PR+RT	184RT+196RT+12PR	0.867	0.547	0.705	0.0004
Week 12 PR	12+63	0.809	0.50	0.626	0.000003
Week 12 RT	184+207	0.603	0.698	0.306	0.007
Week 12 PR+RT	178RT+12PR+72PR	0.78	0.56	0.553	0.0002
Week 24 PR+RT	64PR+98RT	0.561	0.968	0.258	0.011

RT, reverse trasncriptase; PR, protease; SE, standard error. {AUTHOR: OKAY?}

regimen, and approximately 56% of patients had the 184V mutation.

Thus we have established that some amino acid sites not included in a list of 'primary' mutations are independently associated with response, while many that are in such lists are not independent predictors. We have also shown that, for this dataset, the use of the classification tree method [13] offered an improvement over multiple regression. The reason for the improvement appears to be that the additional sites individually make a relatively small contribution to the model but may be associated with a large interaction. Thus, amino acid 135 does not even appear in Table 2, however, in the 184M group, classification by amino acid at position 135 increases the frequency of identification of failures in this group by a factor of two. The reason 135 on its own has no predictive value is that most cases of 135I are associated with 184V, whose effect is very large. While this specific observation requires confirmation in a larger dataset, that the CART approach is most successful of the three methods of analysis used may reflect the underlying match between the bifurcation incorporated in this method and the bifurcation process inherent in the evolution of viral genotypes. We note that mutations at amino acid 135 in RT have been shown to have measurable effects on NNRTI suceptibility *in vitro* [14].

The implication of these observations for interpretation of genotype data is that for any given dataset there are probably some amino acid sites which are substantially more important in determining response than other 'primary' sites. This inevitably means that predictions based on number of sites would be less accurate than those that took account of presence of specific mutations. Further, substantial improvements can be made in prediction of response by analyses designed to take account of the large interaction effects between amino acid sites that contribute to determining drug susceptibility phenotype.

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