Longitudinal Use of a Line Probe Assay for Human Immunodeficiency Virus Type 1 Protease Predicts Phenotypic Resistance and Clinical Progression in Patients Failing Highly Active Antiretroviral Therapy

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An observational study assessed the longitudinal use of a new line probe assay for the detection of protease mutations. Probe assays for detection of reverse transcriptase (Inno-LiPA HIV-1 RT; Innogenetics) and protease (prototype kit Inno-LiPA HIV Protease; Innogenetics) mutations gave results for 177 of 199 sequential samples collected over 2 years from 26 patients failing two nucleoside reverse transcriptase inhibitors and one protease inhibitor (first line: indinavir, $n = 6$; ritonavir, $n = 10$; and saquinavir, $n = 10$). Results were compared to recombinant virus protease inhibitor susceptibility data ($n = 87$) and to clinical and virological data. Combinations of protease mutations (M46I, G48V, I54V, V82A or -F, I84V, and L90M) predicted phenotypic resistance to the protease inhibitor and to nelfinavir. The sum of protease mutations was associated with virological and clinical outcomes from 6 and 3 months on, respectively. Moreover, a poorer clinical outcome was linked to the sum of reverse transcriptase mutations. In conclusion, despite the limited number of patients studied and the restricted number of codons investigated, probe assay-based genotyping correlates with phenotypic drug resistance and predicts new Centers for Disease Control and Prevention stage B and C clinical events and virological outcome. Line probe assays provide additional prognostic information and should be prospectively investigated for their potential for treatment monitoring.

Triple-drug therapy including protease inhibitors (PI) results in a sustained viral load suppression and CD4 cell restoration (6). This so-called highly active antiretroviral therapy (HAART) remarkably reduces hospitalization rates, morbidity, and mortality (12). HAART failure is often closely linked to the emergence of drug-resistant virus. The accumulation of mutations in human immunodeficiency virus (HIV) protease (PRO) is normally an ordered process, with at least one active-site mutation occurring first, leading to a less fit virus, followed by the appearance of one or more non-active-site mutations that compensate for the impaired fitness (9). Cross-resistance is widespread among PI (4), possibly with the exception of the newer compounds amprenavir (APV) (13) and lopinavir.

Observational studies have shown that sequence-based genotyping or recombinant virus phenotyping can predict virological response to second-line therapy in addition to drug history and viral load measurements (7, 21). Resistance tests have also proven their short-term clinical relevance in intervention-based trials (5). Nevertheless, longitudinal data on HIV genotype, phenotype, and viral or immune markers in initially PI-naïve patients are still limited.

A commercialized line probe assay (Inno-LiPA HIV-1 RT; Innogenetics, Belgium) detects mutations related to nucleoside reverse transcriptase inhibitor (NRTI) resistance despite a certain number of hybridization failures due to a limited number of DNA probes in the assay (7.3%) (19) and gives highly concordant results with direct sequencing (15). More recently, a new LiPA became available for the detection of PI-related resistance mutations. This assay was previously compared to direct sequencing in longitudinal clinical samples. The comparison of results from both assays revealed rare (<1% of analyzed codons) major discrepancies (i.e., pure mutant result in one assay and pure wild-type result in the other). LiPA detected transient mixed virus populations containing I84V, M46I, G48V, and L90M earlier and more frequently than sequencing (16).

In the present work, we investigated the longitudinal use of LiPA for PRO and reverse transcriptase (RT) in patients starting PI-based HAART and subsequently experiencing treatment failure. Sequential patient samples were analyzed in batch-testing to study the association between mutations and either phenotypic PI resistance or treatment outcome.

MATERIALS AND METHODS

Patients and treatment. Twenty-six patients monitored at the Centre Hospitalier de Luxembourg for a mean duration of 2.5 ± 0.6 years were retrospectively included in an observational study because they had experienced virological failure after an initial treatment response. Failure was defined by a mean rebound to within 1 $\log_{10}$ RNA copies/ml after an initial response. Baseline data were obtained at the time of initiation of the first PI. The majority of patients had advanced disease (Centers for Disease Control and Prevention [CDC] clinical stage C [2], $n = 14$; CDC CD4 stage 3, $n = 21$), and half of the patients had prior exposure to NRTIs (median number of drugs, 2). The mean baseline viral load was 4.41 ± 0.90 $\log_{10}$ copies/ml. A total of 22 patients were infected with clade B virus.

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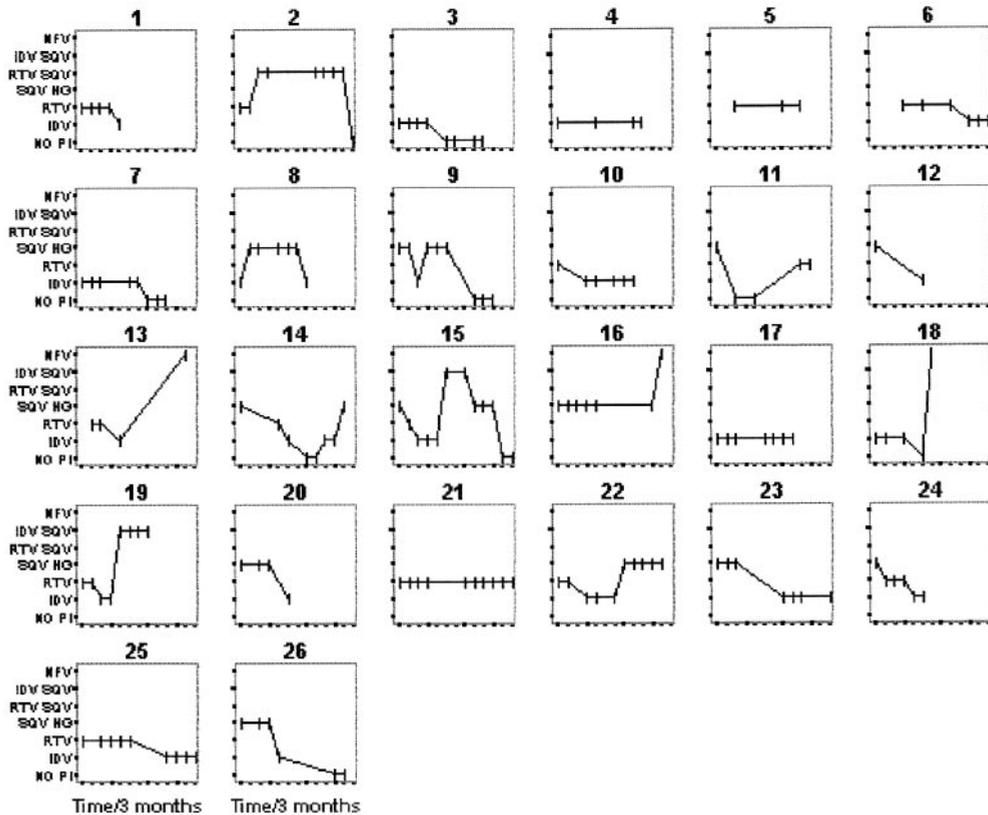
FIG. 1. PI therapy (one graph per patient, with time [3-month intervals, 36-month follow-up] on the *x* axis). Vertical bars indicate when samples were obtained. HG, hard gel.

Nucleic acid extraction and LiPAs. Viral RNA extraction and LiPA were done as described previously (16, 19) and were able to detect wild-type enzymes as well as those with the PRO mutations D30N, M46I, G48V, I50V, I54V, V82A or -F, I84V, and L90M and the RT substitutions M41L, T69D or -N, K70R, L74V, M184V, L214F, and T215Y or -F. Codon 90 of the PRO was analyzed on a separate LiPA strip. Operators performing and interpreting LiPA were blinded for both clinical and phenotypic susceptibility data. The test was done on the same material used for viral load and recombinant virus.

Recombinant-virus assay (RVA). A replicating virus was obtained through homologous recombination of a PRO-deletion-containing, HIV-1$_{HXB2}$-derived provirus (Glaxo Wellcome Research and Development, Hertfordshire, United Kingdom) with a nested-PCR product fragment from a clinical isolate. Primers and assay conditions were previously described (17, 20). Briefly, HIV RNA was reverse transcribed with primer RT02. A 2,220-bp fragment containing PRO and RT was generated by using the primers AV150 and RT02. A nested PCR using inner primers RVP5 and RVP3 produced a 643-bp fragment containing the PRO coding regions of *pol*. After electroporation, stocks of chimeric viruses were harvested from the culture supernatant as cytopathic effect appeared. The recombinant virus was titrated for infectivity and used in a drug susceptibility assay. Briefly, 7 threefold dilutions of drugs were tested in triplicate. The median 50% inhibitory concentration was calculated with the median-effect equation (3). Results were expressed as resistance compared to wild-type HIV-1$_{III-B}$. Indinavir (IDV) was provided by Merck (West Point, Pa.), ritonavir (RTV) was provided by Abbott (Abbott Park, Ill.), saquinavir (SQV) was provided by Roche (Welwyn, United Kingdom), nelfinavir (NFV) was provided by Agouron (San Diego, Calif.), and APV was provided by Glaxo-Wellcome (Erembodegem, Belgium).

Plasma viral load. HIV-1 plasma viral load was measured by a second generation branched DNA assay (Quantiplex 2.0; Chiron, Cergy-Pontoise, France) with a detection limit of 500 copies/ml. Isolates with undetectable viral loads were retested with the ultrasensitive Quantiplex 3.0, able to detect 50 copies/ml.

Predictor variables. Mutation-related predictors were time-dependent variables, assessed every 3 months. The quasispecies phenomenon allowed a scoring system for mutations ranging from 0 (wild type) to 1 (mutant). Wild-type–mutant mixtures were scored as 0.5. Other possible predictors were baseline characteristics, based on a single measurement (see Results). Compliance could not be reliably evaluated with previously recorded data.

Statistics. The association between predictor and longitudinal dependent variables was measured by the slope coefficient of a linear mixed-effects regression (10). A random-effect model was fitted with first-line PI assignment as a fixed effect and the other predictors as covariates. For a given patient, each time point was weighted inversely proportionally to the total number of time points measured for that patient, thus equaling the total statistical weight assigned to each patient monitored longitudinally. The PRO mutations were analyzed for their association with phenotypic resistance. Significant predictors ($P < 0.05$) from the univariate analysis were included in multivariate models. The key PRO mutations were determined as significant and independent predictors of resistance to at least one PI in multivariate analyses. All calculations were made with SPSS 9.0 statistical software (SPSS, Chicago, Ill.).

RESULTS

Treatment and samples. Initial HAART regimens were based on one PI (Fig. 1) and two NRTIs. During the study period, patients changed PI (e.g., NFV) or ceased PI therapy (for no additional therapy or for nevirapine) (Fig. 1). Resistance testing was not used to guide therapy during the study. Blood samples for viral load and CD4 cell counts were taken between April 1995 and February 1999, during the 3-monthly clinical evaluations and whenever a clinical event occurred. A total of 177 of 199 samples were evaluated retrospectively with the Inno-LiPA HIV Protease kit (prototype kit) and the Inno-LiPA HIV-1 RT kit (19). A limited number of samples ($n = 134$), collected every 3 to 6 months, were subjected to phenotypic PI resistance testing for IDV, RTV, SQV, and NFV. Only
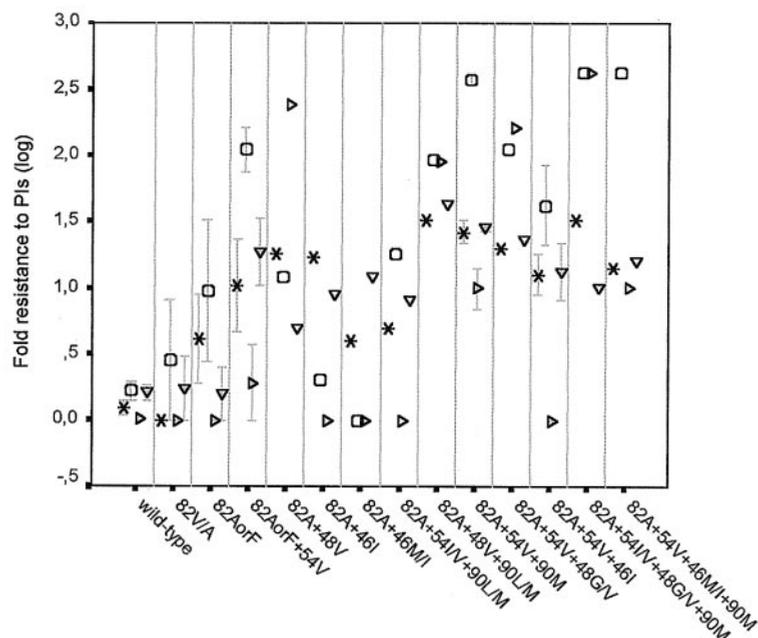
FIG. 2. Association between mutation patterns based on M46I, G48V, I54V, V82A or -F, and L90M and phenotypic resistance for PI (log$_{10}$ resistance [fold]). Values are means; error bars are shown when several phenotypic resistance values correspond to one mutational pathway and indicate 1 standard error of the mean. ∗, IDV; ○, RTV; ▷, SQV; ▽, NFV.

isolates at least fourfold resistant to IDV, RTV, and SQV ($n = 32$) were retested later for APV, although this drug was not used during the study period.

Drug resistance pathways and phenotypic drug susceptibility. The RVA gave 87 of 134 interpretable results (median = 3 per patient, quartiles 2 to 6.5). LiPA PRO yielded 20 genotypic correlates of phenotypic resistance. D30N was not detected in NFV-resistant isolates. A phenotypic resistance profile could be deduced from the combinations of mutations but displayed some variation. Figure 2 shows these patterns excluding I84V, which did not contribute to the correlates.

In linear mixed-effect regression, V82A or -F and I54V were individually and statistically associated with increased phenotypic resistance to the four PI (log$_{10}$-transformed resistance [-fold] normally distributed), as G48V was for SQV and M46I was for both IDV and RTV. I84V was not linked with resistance magnitude (not shown). L90M was a poor predictor of NFV and SQV resistance in the study cohort. The sum of key PRO mutations, adding all mutations except I84V, was related to the resistance level for the four PI (slopes: IDV, 0.487 ± 0.079; RTV, 0.752 ± 0.129; SQV, 0.410 ± 0.121; NFV, 0.464 ± 0.068; $P \leq 0.002$ for all the analyses). In multiple regression, V82A or -F and I54V were independently associated with an increase in IDV and RTV resistance magnitude, as I54V was for NFV and G48V and I54V were for SQV (not shown).

Stepwise regressions were subsequently performed, but only at virus level (without considering the patient factor), with a forward analysis based on a log-likelihood ratio with the probability for stepwise entry set to 0.05. V82A or -F and I54V could account for 76% of variance in RTV resistance, and V82A or -F added to the predictive value of G48V for SQV resistance. Cross-resistance to NFV rose with mutations located at codons 82, 46, 48, and 54 (Tables 1 and 2).

The presence of at least any two of V82A or -F, I54V, M46I, and L90M predicted resistance (≥8-fold) to RTV, whereas G48V and L90M were associated with resistance (≥8-fold) to SQV, IDV, and RTV and, for 75% of samples, with NFV (Table 1). However, the pathways were overlapping. The absence of any mutation from the two previous patterns was a good predictor of drug susceptibility (<4-fold).

I54V was always associated with V82A or -F. Compared to pure wild type, the I54V–wild-type mixture yielded a significantly lower susceptibility to IDV, RTV, and SQV (e.g., for RTV, mean square = 9.55, F ratio = 35.46, $P = 0.001$). The same applied to codon 82 and RTV susceptibility (data not shown).

The sum of key PRO mutations was associated with the extent of cross-resistance (the number of PI [maximum of four] associated with a resistant phenotype, i.e., ≥8-fold resistance, scored as 1, or an intermediate phenotype, i.e., 4- to 8-fold resistance, scored as 0.5) (slope = 1.056 ± 0.154, $P < 0.001$).

TABLE 1. Association between protease mutations and resistance phenotype

Positions and mutation[a]	n	Phenotype (fold resistance)	% of strains resistant to:			
			IDV	RTV	SQV	NFV
82, 54, 46, 90						
Mutation at ≥2	8	R (≥8)	63	100	38	67
Mutation at none	24	S (<4)	91	85	100	79
48, 90						
Mutation at ≥1	11	R (≥8)	100	100	100	75
Mutation at neither	35	S (<4)	63	55	84	68

[a] Mutations: M46I, G48V, I54V or -T, V82A or -F, and L90M.

TABLE 2. Contribution of specific mutations to
variance in resistance magnitude[a]

Mutation location(s)[b]	% of variance in resistance magnitude for:			
	IDV	RTV	SQV	NFV
82	57	66		30
82, 46				37
82, 46, 48				44
82, 46, 48, 54				54
82, 54		76		
48			52	
48, 82			63	
48, 82, 46			67	
48, 82, 46, 54			70	

[a] Stepwise multiple regression was used to measure the contribution of specific mutations to the percentage of variance (R^2) in the resistance magnitude ($\log_{10}$ fold resistance). Mutations were scored as 1 when exclusively present, 0.5 when present in combination with the wild type, and 0 when absent. For example, V82A or -F was entered first and accounted for 66% of the variance in RTV resistance magnitude; I54V was entered second and accounted for a further 10%.
[b] Mutations: M46I, G48V, I54V or -T, V82A or -F, and L90M.

Mutations associated with cross-resistance were I54V (slope = 1.180 ± 0.240, $P < 0.001$) and V82A or -F (1.153 ± 0.279, $P = 0.001$), in contrast to L90M (0.645 ± 0.495, $P = 0.215$).

Four of 32 samples showed >4-fold resistance to APV (data not shown). All four patients with APV-resistant virus had been treated with RTV or SQV before. None of these viruses had the APV-specific mutation I50V, but they harbored V82A, I54V ($n = 4$), G48V ($n = 1$), and I84V ($n = 1$). The sum of key PRO mutations was associated with the magnitude of APV resistance (slope = 1.54 ± 0.538; $P = 0.031$).

LiPA results and treatment outcome. Virological and immunological outcomes were the $\log_{10}$-transformed changes of viral load or CD4 cell counts compared to baseline values. Viral loads reported as "less than 50 copies/ml" were set to 25 copies/ml, as previously suggested (6). The number of new stage B and C events (2) during follow-up defined clinical outcome. A change of stage, considered a stronger marker, was double-scored. Various models were built in which end points were moved forward at fixed intervals of 3, 6, 9, or 12 months. The reported model evaluates the shortest interval for which significance was reached between the time-dependent predictor and the end point. The first time point of longitudinal outcome variables was set at the end of this interval; e.g., in the prediction model from 6 months on, the baseline viral genotype was assessed for its association with the end point at month 6, as was the genotype at month 3 with the end point at month 9 for the same patient, and so forth for each individual.

Baseline CD4 count ($\log_{10}$ cells/μl) was associated with a poorer virological response from 6 months on (Table 3). Baseline characteristics, such as a higher clinical stage (scored from 1 to 3) or a higher number of NRTI resistance mutations, were linked to a higher number of new clinical events from 3 months on. The number of NRTI mutations was a stronger predictor than the NRTI history factor (either the number of drugs or the duration of prior exposure). In contrast, viral load (either continuous [$\log_{10}$ copies/ml] or ordinal [≥ 5 or < 5 $\log_{10}$ copies/ml]) and the choice and duration of the initial PI were not predictors (data not shown).

LiPA results predicted treatment outcome in longitudinal analysis. Indeed, there was a relationship between a higher number of key PRO mutations and poorer virological and clinical outcomes (Table 3), even after having controlled for the above confounders.

Of eight patients, with no baseline RT mutations, developing failure linked to resistance mutations, four displayed RT without PRO mutations and a response to second-line therapy: two had the T215T/Y mutation after receiving zidovudine (ZDV), zalcitabine (ddC), and SQV; one had the M184V mutation after receiving lamivudine (3TC), ddC, and SQV; one had the K70K/R mutation after receiving ZDV, 3TC, and IDV. One patient had the M184V after receiving 3TC, ddC, and RTV before the PRO mutations, and another had a T215T/Y mutation after receiving ZDV, 3TC, and SQV before the PRO L90M.

Baseline characteristics such as CD4 cells and duration of prior NRTI treatment were predictors of immunological outcome from 9 months on, in contrast to genotypic scores (not shown).

DISCUSSION

Our study suggests that PRO mutations detected by the new LiPA correlate with phenotypic drug resistance. The correlation is generally similar to what has been reported by others using sequencing. In general, the number of mutations is linked to the degree of PI resistance (11). However, LiPA-based genotype seems to correlate with a broader range of

TABLE 3. Association of virological and clinical outcomes
with resistance mutations[a]

Predictor	Slope (P^b) for:	
	Virological outcome[c]	Clinical outcome[d]
Univariate analyses		
Baseline clinical and biological characteristics		
CDC clinical stage (1–3)	0.085 (0.663)	0.606 (**<0.001**)
CD4 cell counts ($\log_{10}$ cells/μl)	−0.800 (**0.009**)	−0.156 (0.081)
Number of prior NRTIs	0.080 (0.853)	0.500 (**0.001**)
Sum of key RT mutations at baseline	2.306 (**0.001**)	2.000 (**<0.001**)
Sum of key PRO mutations[e] (3-monthly)	0.348 (**0.013**)	0.235 (**<0.001**)
Multivariate analyses		
Sum of key PRO mutations	0.337 (**0.020**)	0.159 (**<0.001**)
Sum of key RT mutations[e] at baseline	0.214 (0.690)	0.807 (**0.003**)
Baseline CD4 cells count ($\log_{10}$ cells/μl)	−0.823 (0.061)	
Baseline CDC clinical stage (1–3)		0.556 (**<0.001**)

[a] The association between predictors and end points was measured by the slope coefficient of a weighted linear mixed-effect regression. Predictors were baseline patient characteristics or the sum of drug resistance mutations (95 samples from 26 patients, one per supplementary mutation; mixtures were scored as 0.5). End points were virological and clinical outcomes. Separate univariate models identified significant predictors (i.e., confounders). P values were further adjusted in multivariate analyses for the confounders.
[b] Values less than 0.05 are in bold.
[c] $\log_{10}$-transformed changed compared to baseline values, from 6 months on.
[d] Number of new CDC stage B and C events (a change of stage was double-scored), from 3 months on.
[e] Key PRO mutations: M46I, G48V, I54V, V82A or -F, and L90M (time-dependent predictors).

phenotypes compared to sequencing (18). Indeed, LiPA may detect minorities of emerging mutant or residual wild-type virus (16). Furthermore, Inno-LiPA HIV Protease lacks certain non-active-site mutations, such as A71V or -T and L10I or -V. In addition, phenotypic assays have their own limitations due to their variability, sensitivity, precision, and accuracy. Moreover, it would be useful to compare the data of this RVA with those of other phenotypic assays. A specific limitation of this assay is that none of the PRO cleavage sites are cloned into the vector, whereas they might be required for optimum detection of resistance in the RVA, though virus fitness can remain compromised even in the presence of these compensatory cleavage site mutations (14). Until accurate measurements of viral fitness, genotypic heterogeneity, and formulation of drug pressure are available, we have to rely on statistical approaches despite treatment heterogeneity and patient variation. Nonetheless, mixed populations seem to correlate, in our study, with higher levels of resistance than pure wild type, indicating that phenotypic resistance might occasionally proceed from an averaging of wild-type–mutant population susceptibility.

LiPA results predict treatment outcome. The accumulation of PRO mutations, progressively detected in patients treated with largely cross-resistant compounds, appear to predict a poorer virological response from 6 months on. There is a direct relationship between the number of mutations and the degree of poor response, as emphasized by a similar relationship with in vitro drug resistance. In different settings of salvage therapy, previous studies have shown that sequence-based genotyping predicts in vivo cross-resistance (1, 5, 7, 21). In first-line therapy, regular resistance testing might become a standard in patient care, helping to avoid unnecessary drug exposure and to adapt treatment before high levels of resistance are reached, which may take several months under suboptimal virus suppression (11). A delay in plasma HIV RNA clearance should be considered an indication for resistance testing, after correct drug exposure has been addressed. Early detection of emerging mutations with a simple assay may be suitable in such situations. In addition, HAART based on PI selected first RT mutations in some NRTI-naïve patients, in agreement with previous studies (8). Some of them, detected as mixtures, are ZDV related.

Probe assay genotyping is a predictive tool for a more rapid clinical progression independently of the initial disease stage in our patients with advanced disease. The detection of emerging mutations as minor variants may contribute to the prediction of clinical events. In contrast, resistance mutations were not associated with a poorer immunological outcome in our deeply immunosuppressed patients.

Intervention-based prospective trials are needed to evaluate the clinical utility of repeated LiPA testing. They should also address whether early detection of minor variants has a clinical repercussion. In addition, assays for non-NRTI drugs are needed.

Our study has the limitations of the longitudinal feature, which reduces patient sample size and complicates analyses. Statistical inferences have to be confirmed on a larger patient sample. Genotypic scores only give an approach to the understanding of resistance in the clinical field. Moreover, these scores are useful only in the setting of largely cross-resistant compounds where resistance is related to the accumulation of shared mutations. Correlation between mixed genotype and phenotype with intermediate resistance must be interpreted carefully, as the PCR used in these assays could select for specific variants. Moreover, observational studies rarely account for drug level and adherence monitoring. The absence of a control group is handled by the comparison of mutant versus wild-type sequences at virus and patient levels.

In conclusion, despite a limited number of patients and a restricted number of codons investigated, genotyping based on probe assays predicts new CDC stage B and C clinical events from 3 months on and viremia from 6 months on. There is also a significant association of LiPA genotype with PI resistance phenotype, suggesting that treatment outcome is related to the progressive accumulation of PRO mutations. The longitudinal use of probe assays provides additional prognostic information for patients developing first-line treatment failure and should be evaluated prospectively for its potential to optimize monitoring of therapy and to prevent emergence of broadly cross-resistant virus.

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