A Survival Method to Estimate the Time to Occurrence of Mutations: An Application to Thymidine Analogue Mutations in HIV-1–Infected Patients

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Virologic studies of human immunodeficiency virus type 1–infected patients have investigated either the emergence of resistance mutations according to the treatment received (type I) or their effect on subsequent regimens (type II). Type I studies provide an estimation of the frequency distribution of mutations for a given duration of therapy, but the delay to emergence of these mutations cannot be assessed. We suggest using a nonparametric estimator that generalizes the Kaplan-Meier method to data from type II studies to estimate the time to occurrence of mutations. Patients had no treatment interruption before viral genotyping. Although the curves should be interpreted with caution, they provide useful information about the kinetics of the emergence of mutations. The method was applied to the emergence of thymidine analogue mutations in patients previously treated with zidovudine (ZDV) plus didanosine or zalcitabine. Although K70R has been described as the first mutation to appear in patients receiving ZDV monotherapy, the T215Y/F mutation appeared first in patients receiving dual-nucleoside combination therapy.
characteristics of mutation data lead to cautious interpretation of such curves because of the uncertainty in the actual time to occurrence of the mutations. Indeed, mutations occurred before viral genotyping was performed. We also estimated the time to occurrence of at least 1, at least 2, at least 3, and at least 4 mutations. Methods were discussed and were applied to patients previously treated with zidovudine (ZDV), zalcitabine (ddC), and didanosine (ddI) in estimating time to occurrence of thymidine analogue mutations (TAMs).

**PATIENTS AND METHODS**

**Patients.** Patients in the present study were enrolled in the NOVAVIR (Agence National de Recherche sur le SIDA [ANRS] 073) trial, and informed consent was obtained from all patients at the beginning of the NOVAVIR trial. NOVAVIR is a randomized open-label trial that compares therapy with stavudine (d4T) versus ZDV each in combination with lamivudine (3TC) plus indinavir (IDV) in HIV-1–infected patients pretreated with ZDV, ddI, and/or ddC, but inexperienced for d4T, 3TC, and protease inhibitors (PIs) [11]. Patients enrolled in the present study had documented HIV-1 infection, as determined by a positive ELISA result for HIV infection that was confirmed by Western blot, were aged ≥18 years, and had >6 months of previous ZDV, ddI, and/or ddC cumulative treatment, either as monotherapy or as combination antiretroviral therapy (ART). Patients had HIV-1 plasma RNA levels of 5000–200,000 copies/mL. Exclusion criteria included previous treatment with zidovudine (ZDV), stavudine (d4T), and didanosine (ddI) in estimating time to occurrence of at least 1, at least 2, at least 3, and at least 4 mutations. RT codons 35–260 were analyzed. TAMs were defined as M41L, D67N, K70R, L210W, T215Y, or F, and K219Q or E.

**Statistical methods.** First, we were interested in the estimation of the delay to occurrence of a specific mutation among TAMs. The methodology used 2 variables: the duration of previous therapy and an indicator variable representing presence or absence of the mutation. Both variables were recorded at study entry of the NOVAVIR trial. In our analysis, the duration of previous therapy was considered as the “survival time,” and presence or absence of the mutation was the censoring indicator. Patients without the mutation at study entry were considered to be “right censored,” because the actual, but unknown, times to occurrence of the mutation were greater than the observed durations of previous therapy (right-censored survival time). Patients with the mutation were considered to be “left censored,” because the actual times to occurrence of the mutation for these individuals were less than that for observed durations of previous therapy. A nonparametric maximum likelihood estimate that generalizes the Kaplan-Meier for such data has been proposed in the context of interval-censored data [13–15].

Consider that the time to occurrence of the mutation \( X \) for the \( i \)th individual can be right censored or left censored, as described above. Let \( N_i \) and \( N_i^l \) denote the number of individuals who are right censored and left censored, respectively. In the present study, we were interested in estimating the cumulative distribution function \( F(x) = P(X_i < x) \) from such data. The distribution function was related to the survival function \( S(x) \), with \( S(x) = P(X_i > x) = 1 - F(x) \). The likelihood can be written as follows:

\[
L(F|D) = \prod_{i=1}^{N_i} F(X_i) \times \prod_{i=1}^{N_i^l} [1 - F(X_i)] ,
\]

where \( D \) denotes the observed data.

The nonparametric maximum likelihood estimator (NPMLE) \( F \) of \( F \) can be obtained by maximizing the log-likelihood using the EM algorithm [13, 14]. For a given level of censoring, estimates of the distribution function with only left-censored and right-censored data are obviously less accurate than Kaplan-Meier estimates that are applied to data, including exact survival time and right-censored data. Confidence intervals (CIs) were obtained by use of bootstrap techniques (\( n = 500 \) samples).

The method was only applied to the group of patients who began ART with a dual-nucleoside combination. The reasoning is as follows: (1) these patients received similar treatment regimens, and occurrence of mutations were closely related to the treatment received and to the sequence of treatment received [16]; (2) 2 distinct mutational patterns have been identified in the 2 groups of patients [16]; and (3) several patients in group
Figure 1. Frequency distribution of thymidine analogue mutations (TAMs), according to the initial regimen received. Data are percentage of patients carrying mutations at reverse-transcriptase codons 41, 67, 70, 210, 215, and/or 219. Nos. above bars indicate no. of isolates included in each category. ddI, didanosine; ddC, zalcitabine; ZDV, zidovudine.

2 had a previous duration of therapy $>8$ years that led to a great uncertainty in the time to emergence of the mutations. We used a nonparametric test for use with discrete failure times to compare distribution function between groups of patients [17].

We also estimated the time to occurrence of at least 1, at least 2, at least 3, and at least 4 TAMs. Patients with a lower number of mutations than the distribution being estimated were considered to be right censored at the time of duration of previous therapy, whereas other patients were considered to be left censored. For example, patients with $\leq 1$ TAM were considered to have right-censored observations for estimating the distribution of the time to occurrence of at least 2 mutations.

RESULTS

Baseline characteristics. Blood samples for virus isolation were obtained at study entry for 155 patients randomized in the NOVAVIR trial [10]. Ninety patients had begun to receive ART with a dual-nucleoside combination (group 1) of ZDV plus ddI ($n = 57$) or ZDV plus zalcitabine ($n = 33$), whereas 50 patients had started with ZDV monotherapy and then added ddI or ddC (group 2). The 15 remaining patients received both dual-nucleoside combinations or ZDV alone.

Comparison between patients in group 1 and patients in group 2 has been investigated in depth, as described elsewhere [16]. Patients in group 2 had begun ART in the era of ZDV monotherapy and had a much longer median duration of previous therapy than patients in group 1 (38 and 14 months, respectively; $P < .0001$). There was no statistically significant difference in the number of TAMs between patients in group 1 and patients in group 2 (mean, 2.68 vs. 3.06; $P = .13$). The survival methods to estimate time to occurrence of TAMs were applied only to patients in group 1. Therefore, in the present study, results only reflect the 90 patients who began ART with a dual-nucleoside combination.

The most frequent mutations were at RT codons 215 (79%), 41 (51%), 67 (48%), and 70 (40%), whereas mutations at positions 210 (27%) and 219 (23%) were less frequent. Isolates from 79 (88%) of 90 patients had $\geq 1$ TAMs (figure 1), and patients with longer duration of therapy had significantly more TAMs (figure 2; $P = .001$, Kruskal-Wallis test). No significant difference in the number of TAMs ($P = .23$) and in the median CD4 cell count ($P = .27$) were found between patients receiving ZDV plus ddI and patients receiving ZDV plus ddC.

Mutation profiles, according to duration of previous therapy. Figure 3 compares the distinct mutation profiles of the presence of TAMs, according to the duration of previous therapy. Mutations at positions 41, 67, and 70 have a similar mutational pattern, which corresponded to a progressively increasing percentage of mutations with increasing duration of therapy. After only 9 months of ART with a dual-nucleoside combination,
mutated at codon 215 was present in 59% of isolates, and a large majority of isolates carried this mutation after 14 months of ART. Mutation at position 219 occurred late in these patients, and the frequency of mutations at codon 210 was stabilized to ~35% after 14 months of ART.

**Time to occurrence of TAMs.** Figure 4 displays the time to occurrence of each TAM for the 90 patients receiving ZDV plus ddI or ZDV plus ddC. For simplicity of presentation, 2 series of 3 TAMs were grouped on the same graph. Mutations at codon 215 appeared first, with the smallest time to occurrence, because 50% of patients carried this mutation after 7 months (95% CI, 5–8) of ART with a dual-nucleoside combination. Median times to occurrence were 17 (9–24), 20 (8–24), 20 (17–25), 33 (20 to ∞), and 22 (20–57) months for mutations at codons 41, 67, 70, 210, and 219, respectively. Nonparametric comparisons between patients receiving ZDV plus ddC and patients receiving ZDV plus ddI (data not shown) did not provide any statistically significant difference in the time to occurrence of any TAMs, although the unbalanced and relative small sample sizes (n = 33 and n = 57, respectively) limited such findings. The validity of such tests is described below.

The median time of previous therapy to occurrence of at least 1 or at least 2 mutations was 7 months (95% CI, 5–7 and 5–9, respectively; figure 5). The median duration of ART with a dual-nucleoside combination to reach and maintain ≥3 and ≥4 mutations in at least 50% of patients was estimated to be 11 months (7–15 months) and 24 months (22–33 months), respectively. As described above, no statistical difference was found between patients receiving ZDV plus ddI or patients receiving ZDV plus ddC.

**DISCUSSION**

In the present study, we used a nonparametric maximum likelihood estimator that generalizes the Kaplan-Meier to estimate the delay to occurrence of TAMs for patients receiving ART. The methodology considers patients with and without the mutation as left-censored and right-censored observations, respectively. Because patients received the same regimen, they should have a distinct duration of therapy.

Survival methods provide an original way to analyze genotypic data when patients have a range of durations of therapy. Genotypic data recorded at study entry is a genuine example of left-censored data, because the actual time to occurrence of any TAMs, although the unbalanced and relative small samples sizes (n = 33 and n = 57, respectively) limited such findings. The validity of such tests is described below.

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Figure 3. Frequency distribution of each zidovudine-resistance mutation, according to quartiles of duration in months of previous antiretroviral therapy (ART).
Figure 4. Distribution function of the probability to occurrence of each thymidine analogue mutations (M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E) in patients receiving zidovudine plus didanosine or zidovudine plus zalcitabine.

censored observation; if $R_i = \infty$, then we have a right-censored observation. In the present study, interval-censored data would correspond to viral genotyping at distinct time points during the follow-up. In this situation, the accuracy of the estimate depends markedly on the length of intervals of periodic viral genotyping, and such data would greatly improve estimates of the time to occurrence/presence of mutations. However, this situation was unlikely to occur, because viral genotyping requires high viral replication that corresponds to virologic failure that usually precedes treatment changes for patients. Few studies recorded multiple genotyping data, because their sample sizes were small [1, 2] or their studies required specific limits of detection [3]. Left-censored methods with a larger genotypic database would be helpful to confirm our findings, as well as to distinguish among distinct variants defined as mutants in certain positions. For example, in the present study, we could not distinguish between T215Y and T215F or K219Q and K219E. ART with a dual-nucleoside combination is no longer
Figure 5. Distribution function of the probability to occurrence of at least 1, at least 2, at least 3, and at least 4 thymidine analogue mutations (TAMs) in patients receiving zidovudine plus didanosine or zidovudine plus zalcitabine.

used as the current antiviral regimen, and the order of appearance of mutations could not be extrapolated to highly active ART or other ARVs with dual-nucleoside combinations. The methods described in the present study can be applied to current treatment regimens and might be useful as a secondary end point to compare treatment arms or strategies in clinical trials.

Analysis was applied only in the group of patients beginning ART with dual-nucleoside combination for ease of data interpretation. Another study [16] indicated that patients beginning with ZDV monotherapy had more mutations, compared with that for the other group of patients, although the difference was not statistically significant ($P = .13$). Of importance, the authors suggested 2 distinct mutational patterns according to the initial treatment received (ART with a dual-nucleoside combination or ZDV monotherapy). Patients who started ART with a dual-nucleoside combination had shorter duration of previous therapy, compared with that for patients beginning with ZDV monotherapy. Half the patients who began with ZDV monotherapy had $\geq 38$ months of previous therapy, which corresponded to a great uncertainty in the time to occurrence/presence of the mutations. This method implies that the proportion of occurrence to mutations is increasing monotonically and that, without any treatment interruption, there is no phenomenon disappearance of a mutation. This phenomenon has been described for the K70R mutation [1]. However, this phenomenon has been described only in 1 study and, as indicated by its authors, may be explained by an apparent disappearance caused by the balance of different components of the virus population. The minimum detection of the virus population was 20% in the NOVAVIR trial.

Different situations can provide bias in the estimates or in the group comparisons. Patients should have no treatment interruption before viral genotyping, because, without the pressure of treatment, wild-type virus returns as the dominant virus. A period of treatment interruption for some patients will tend to underestimate the time to occurrence/presence of the mutations under investigation. In the present study, patients had no treatment interruption period before viral genotyping at study entry. Patients who had incomplete adherence to the regimen and consequently did not develop mutations also tend to underestimate the curves of the presence of mutations. In the NOVAVIR trial, we showed that patients who had zero or few ZDV mutations at study entry probably had difficulties in medication adherence [10]. Another difficulty will appear if there is a discrepancy in medication adherence between the groups of patients being compared. For instance, a comparison between patients receiving ART with a dual-nucleoside combination or ART including a PI being administrated every 8 h and without food will potentially lead to a difference in medication adherence. As stated above, a comparison would not be valid in this situation. Another difficulty is the so-called survivor effect that implies that only patients alive at the start of the study may be enrolled in that study. If the rapid development of mutations is somewhat related to the death of patients from previous therapy, this will underestimate the time distribution of the presence of mutations.

A major difficulty for investigating the presence of mutations
in patients with highly suppressed viral replication is that genotyping is very difficult or nearly impossible with standard sequencing methods. Ultrasensitive sequencing procedures when virus load is <400 or 50 copies/mL are available, but mutant viral populations that are observed when viral replication is very low are not fully predictive of the substitutions present at time of viral rebound [18]. Genotyping on viral DNA and searching for archived viral mutant populations could be of interest, but stored peripheral blood mononuclear cells are not always available. Consequently, mutations are usually found in patients experiencing virologic failure. However, in clinical practice, this is not considered to be an obstacle, because we are concerned with the investigation of resistance mutations for subsequent treatment options only in these patients. A bias can appear if the delay between time to virologic failure and time to viral genotyping is quite different among patients or among groups of patients. In the present study, duration of previous therapy was the same between patients receiving ZDV plus ddI and patients receiving ZDV plus ddC (median duration, 13.8 vs. 13.5 months; \( P = .93 \)), but those who received ZDV plus ddI had lower HIV-RNA levels (4.34 vs. 4.49 log units; \( P = .06 \)) and higher CD4 cell counts (313 vs. 265 cells/mm\(^3\); \( P = .27 \)), compared with patients who had received ZDV plus ddC. HIV RNA levels cannot be used to determine the time of delay to virologic failure, because levels of virologic rebound have been shown to be related to mechanisms of failure [19, 20]. Although not statistically significant, higher CD4 cell counts might indicate that patients who had received ZDV plus ddC had a greater delay from virologic failure and viral genotyping. In this situation, curves for patients in the latter group might be underestimated, compared with those for patients in the other group; therefore, the comparison might not be valid.

ZDV resistance has been first described in patients treated with ZDV monotherapy who developed resistance in a stepwise pattern with the mutation at codon 70 appearing first, although the order of emergence can vary [1, 21]. Our results show that, in patients who received ART with a standard dual-nucleoside combination used in 1994–1996, the pathway of accumulating TAMs is different, with the T215Y/F appearing first. The second mutation to appear was among M41L, D67N, or K70R, whereas the L210W and K219Q/E appeared later. Another study [3] indicated that the K70R mutation appeared after the M41L and T215Y/F mutations in patients receiving ART with a dual-nucleoside combination, although the genotypic techniques were different. Identification of distinct patterns of accumulating TAMs was useful, because previous results from other studies [22–24] suggested that different TAMs profiles could influence the virological response to nucleoside RT inhibitors.

Our results also suggest that accumulation of TAMs in patients who treated with a dual-nucleoside combination is not a linear step. The first 2 mutations appeared almost simultaneously, and a duration of 7 months is required to observe at least 2 TAMs in 50% of patients. An additional median of 11 and 24 months of therapy is needed to develop a third and a fourth mutation, respectively. An explanation would be that the presence of 2 mutations, which are probably among codons 41, 67, 70, and 215, would affect the replicative capacity of the virus.

In conclusion, we suggest an original way to estimate the time to occurrence and presence of mutations under different ARTs by use of interval-censored survival methods. The method takes advantage of the range of duration of previous ART when patients are enrolled in clinical trials. For ART with dual-nucleoside combination, our results indicate that the T215Y/F mutation appeared first, whereas, for ZDV monotherapy, the K70R has been described as the first mutation to appear.

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**References**