High frequency of integrase Q148R minority variants in HIV-infected patients naive of integrase inhibitors

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Background: Integrase positions 148 and 155 represent main determinants of resistance to integrase inhibitors. We assessed the prevalence of minority variants harboring such mutations in integrase-naive HIV-infected patients.

Methods: Two groups of patients were studied: 40 heavily antiretroviral-experienced patients, initiating a raltegravir-based therapy and 51 antiretroviral-naive patients. Allele-specific real-time PCR (AS-PCR) systems, developed for Q148H, Q148R and N155H mutations, were performed at baseline for antiretroviral-experienced patients. Samples from antiretroviral-naive patients were tested with the Q148R AS-PCR assay.

Results: The limits of detection of AS-PCR systems were 0.10, 0.10 and 0.05% for Q148H, Q148R and N155H mutations, respectively. AS-PCR systems were successful in 79 of 91 samples. In antiretroviral-experienced patients, Q148R minority variants were frequently detected (26/32 patients, 81%) at low-level frequency (median = 0.40%), whereas no minority variants exhibiting Q148H or N155H mutation were found. Twenty-four of 26 patients exhibiting Q148R variants were virological responders but four of them displayed a delayed virological response occurring between W18 and W36. Two patients exhibited virological failure under raltegravir, both harboring Q148R minority variants at baseline. However, we did not find any association between the presence of Q148R minority variants and an increased risk of virological failure. Q148R minority variants were also found in 86% of antiretroviral-naive patients, a prevalence significantly higher than that of K103N minority variants (26%).

Conclusion: Q148R variants were frequently detected, always at low-level, in antiretroviral-experienced and naive patients. Although their presence was not consistently associated with virological failure, their impact on long-term viral suppression needs to be further investigated.

AIDS 2010, **24**:867–873

Keywords: allele-specific real-time PCR, HIV, integrase inhibitors, minority resistant variants

Introduction

Raltegravir (RAL), the first U.S. Food and Drug Administration–approved antiretroviral drug of the class of integrase inhibitors, showed potent antiviral activity and was well tolerated in both antiretroviral-experienced and antiretroviral-naive patients [1,2]. Resistance pathways to integrase inhibitors mainly involved integrase

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Received: 30 July 2009; revised: 5 October 2009; accepted: 10 December 2009.

DOI:10.1097/QAD.0b013e3283367796

ISSN 0269-9370 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins

mutations Q148H/K/R and N155H, and less commonly the mutation Y143C/H/R [3]. A single mutation at key positions, Q148 or N155, is sufficient to confer resistance with 20× reduced susceptibility to RAL [4,5].

Numerous HIV quasispecies are present in the plasma of HIV-infected patients [6]. The genotypic resistance tests routinely used in clinical practice only detect mutations that are present in more than 20% of viral populations [7]. However, the role of viral variants present at low-level frequency in plasma has been previously involved in resistance development to different antiretroviral drug classes, including protease inhibitors and nonnucleoside reverse transcriptase inhibitors (NNRTIs) [8–10]. Furthermore, recent studies [11–14] suggested that the presence, at baseline before antiretroviral treatment (ART), of resistant minority variants may be clinically important and increased the risk of virological failure. However, at least one study [15] did not find any impact of the presence of minority variants on the virological outcome.

Here, we assessed the presence of Q148H, Q148R and N155H-mutated minority variants in HIV-infected patients naive of integrase inhibitors.

Patients and methods

Patients

Two groups of HIV-1-infected patients naive of integrase inhibitors were studied. The first group included 40 heavily antiretroviral-experienced patients initiating a RAL-containing regimen. Most of them (90%) were infected with HIV-1 subtype B. All patients (30 men and 10 women) had been extensively treated over a median of 14 years. Thus, at baseline, all the patients harbored multidrug-resistant viruses, exhibiting a median of 19 resistance mutations, including five nucleoside reverse transcriptase inhibitor (NRTI)-resistance-associated mutations, one NNRTI-resistance mutation and 13 protease inhibitor-resistance mutations (Table 1). The optimized background treatment contained a median of two active antiretroviral drugs (range, 0–3) in combination with RAL. Plasma samples were collected at baseline, weeks 12, 24 and 48 of RAL-based regimen.

All patients, naive of antiretroviral drugs, infected with HIV-1 subtype B were included consecutively during the year 2008. This second group was comprised of 51 patients. A single plasma sample was collected at the time of HIV-1 infection diagnosis.

HIV-1 RNA viral load and CD4 T-cell count measurements

Plasma HIV-1 RNA levels were measured with the COBAS TaqMan HIV-1 test (Roche Molecular Systems, Branchburg, New Jersey, USA), with a detection limit of 40 copies/ml. CD4 T-cell counts were determined by flow cytometry using the FACScalibur cytometer (Becton Dickinson, San Jose, California, USA).

Table 1. Antiretroviral-experienced patients' characteristics.

	$N = 40$
Sex [male, *n* (%)]	30 (75)
Age [years, median (IQR)]	47 (43–53)
Transmission group [*n* (%)]	
Homosexual/bisexual men	24 (60)
Heterosexuals	12 (30)
Intravenous drug users	12 (10)
Prior AIDS event [*n* (%)]	18 (45)
Duration of antiretroviral therapy [years, median (IQR)]	14 (13–15)
CD4 cell count [cells/μl, median (IQR)]	151 (43–330)
Plasma HIV-1 RNA [$\log_{10}$ copies/ml, median (IQR)]	4.21 (3.60–4.76)
Resistance mutations at baseline [*n* (IQR)]	
NRTI	5 (5–6.5)
NNRTI	1 (0–2)
Protease inhibitor	13 (11–15)

IQR, interquartile range; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor.

Quantification of integrase-mutated minority variants in plasma samples

In order to be able to detect integrase-mutated variants present in minority proportions in plasma viral population, we developed allele-specific real-time PCR (AS-PCR) assays for the Q148H, Q148R and N155H integrase mutations. All three systems were designed with the presence of primer-target mismatches at the 3′ end of the forward primer, known to impair the efficiency of PCR amplification [16]. Quantification of minority viral populations in plasma was performed as follows: 1 ml of plasma was centrifuged and RNA was extracted (QIAamp Viral RNA Minikit; Qiagen, Valencia, California, USA). Reverse transcription-PCR (RT-PCR) was performed with oligonucleotides IN12 and IN13, as previously described [17]. Serial dilutions of RT-PCR products were performed in 10 mmol/l Tris buffer (pH 7.4) containing 0.1 mmol/l EDTA and 100 ng of herring sperm DNA (Sigma, St. Louis, Missouri, USA) per milliliter. Ten microliters of diluted RT-PCR products were used to perform nonselective and selective amplifications reactions in parallel. Nonselective reactions were performed with the primers designed based on subtype B sequence INT-F (5′-TTCCCTACAATCCCCAAAGTCA), INT-R (5′-TGTCTTAAGATGTTCAGCCTGATCT) and the probe INT(148–155) [5′-(6-FAM) AAAGAAAATTA-TAGGCCAGGTAA(MGBNFQ)]. Only the forward primer was changed for the selective amplifications: INT-F(148Hc)2A1T (5′-TTCCCTACAATCCCCAA AGTATC), INT-F(148R)2A1G (5′-TCCCTACAATC CCCAAAGAGG) and INT-F(155H)F1C (5′-CCAAA GTCAAGGAGTAGTAGAATCTATCC) for the allele-specific real-time PCR (AS-PCR) systems designed for the Q148H, Q148R and N155H mutations, respectively.

The reactions conditions were 1× TaqMan buffer, 3 mmol/l $MgCl_2$, 100 nmol/l each primers, 100 nmol/l TaqMan MGB-probe, 0.5 U of uracil-*n*-glycosylase and 1.25 U of AmpliTaq Gold *Taq* polymerase. The cycling parameters were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 50°C for 1 min.

Patient-derived clones independently harboring Q148H, Q148R and N155H mutations on integrase gene were used as DNA standards. Amplification products issued from the different mutated DNA standards and from a wild-type sequence plasmid were serially diluted 10-fold in 10 mmol/l Tris buffer (pH 7.4) containing 0.1 mmol/l EDTA and 100 ng of herring sperm DNA (Sigma) per milliliter. The latter DNA standards dilutions allowed generating standard curves. The percentage of mutated sequences was calculated as follows: percentage mutated sequences = (the quantity of mutated sequences in the sample/the quantity of total sequences in the sample) × 100.

Quantification of K103N-mutated minority variants in plasma samples

The quantification of plasma viral populations expressing the K103N resistance mutation associated with AAC and AAT codon changes was performed using the AS-PCR K103N assay described by Lecossier *et al.* [9]. DNA standards were prepared by PCR amplification of both wild-type and K103N pNL4-3 HIV-1 plasmids (kindly provided by Dr A.J. Hance, INSERM U941, Paris, France).

Results

Allele-specific real-time PCR systems for Q148H, Q148R and N155H integrase mutations

In order to increase the sensitivity of detection of integrase-mutated variants in plasma, we developed AS-PCR systems focused on relevant integrase mutations at positions 148 and 155. The Q148H AS-PCR system was designed to detect the CAC change involved in approximately 90% of Q148H mutations (S. Fransen from Monogram Biosciences, personal communication).

The capacity of an AS-PCR assay to selectively amplify the mutated sequences, corresponding to the AS-PCR assay discriminative ability, is measured by the difference in the number of amplification cycles required to reach threshold fluorescence (ΔCT) between nonselective and selective primers. The ΔCTs of the different AS-PCR assays were found to be 18, 16 and 12 cycles for the Q148H, Q148R and N155H assays, respectively. To assess the specificity of these assays, we tested samples containing only wild-type sequences. No significant amplification signals were detected with the wild-type clone in all three AS-PCR systems (CT > 40 cycles).

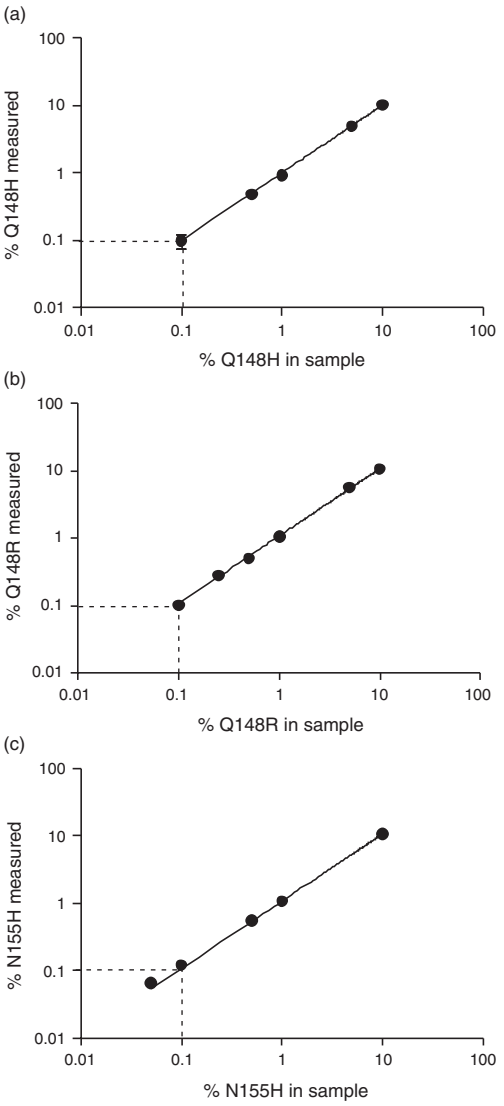

Fig. 1. DNA standard curves for allele-specific real-time PCR assays. DNA standards, one containing the integrase mutation and the other the wild-type integrase sequence were mixed to prepare samples with the indicated percentage of mutated sequences (% in sample). The proportion of mutated sequences was measured (% measured) by AS-PCR as described in the Methods section. (a) Q148H, (b) Q148R and (c) N155H mutation. Results are expressed as mean ± SD obtained in three independent determinations at each point. AS-PCR, allele-specific real-time PCR.

In order to determine the limit of detection of the different AS-PCR assays, the patient-derived mutant clones and the wild-type sequences were mixed in different proportions, and the percentage of integrase-mutated sequences was determined by the AS-PCR assays. Then, we analyzed the relationship between the proportion of mutated sequences measured by the AS-PCR assay and the proportion of mutated sequences added in a given sample (Fig. 1). We showed that the

AS-PCR systems were able to detect Q148H and Q148R variants at levels as low as 0.10% and N155H variants at levels as low as 0.05%.

HIV-1 minority variants in heavily pretreated patients

Immunovirological response to raltegravir-based therapy

At baseline, before initiating RAL-based regimen, median HIV-1 RNA level was 4.21 $\log_{10}$ copies/ml [interquartile range (IQR) = 3.60–4.76] and CD4 T-cell count was 151 cells/μl (IQR = 43–330). CD4 T-cell count increased to 276 (IQR = 136–366) and 281 (IQR = 147–403) cells/μl at weeks 12 and 24, respectively. At week 12, 31 of 40 patients (78%) achieved HIV-1 RNA levels below 40 copies/ml; three patients displayed an early virological failure associated with integrase-mutated virus (previously described in [18]). The six remaining patients had a median HIV-1 RNA level of 2.01 $\log_{10}$ copies/ml at week 12 and reached undetectable HIV-1 RNA levels between weeks 18 and 36 while on the same antiretroviral-based regimen. Overall, 37 patients were virological responders. All patients but one reached the follow-up of week 48. No virological failure occurred between weeks 24 and 48.

Assessment of integrase-mutated minority variants at baseline of raltegravir-based therapy

Virus amplification by the different AS-PCR assays was successful for 32 of 40 samples (80%), and ineffective for eight samples, including five HIV-1 non-B subtypes. Further investigations confirmed that all integrase sequences from the latter samples exhibited primers, probe nucleotides mismatches or both (data not shown).

Among the 32 amplified samples, Q148R minority variants were frequently detected at baseline, found in 26 of 32 samples (81%) (Fig. 2a). The Q148R-mutated variants were always present at low-level frequency (median = 0.40% of total sequences, IQR = 0.29–0.57%). Conversely, we did not detect any Q148H or N155H minority viral populations at baseline of RAL-based therapy.

Impact of the presence at baseline of Q148R minority variants on the virological response to raltegravir-based therapy

Twenty-four (92%) of the 26 patients, exhibiting Q148R minority variants at baseline, were virological responders to RAL-based treatment, reaching HIV-1 RNA plasma levels below 40 copies/ml at week 12 for 20 patients, and between weeks 18 and 36 for the four remaining patients. In all six patients without Q148R variant at baseline, undetectable HIV-1 RNA levels were achieved within the first 3 months of RAL-based treatment. Considering the three patients displaying virological failure under RAL-based therapy, only two can be assessed by AS-PCR assays, both exhibiting Q148R minority variants at baseline. Interestingly, in these patients, the RAL resistance pathway began with the Q148R mutation, detected as the major viral population within the first 3 months of RAL-based therapy.

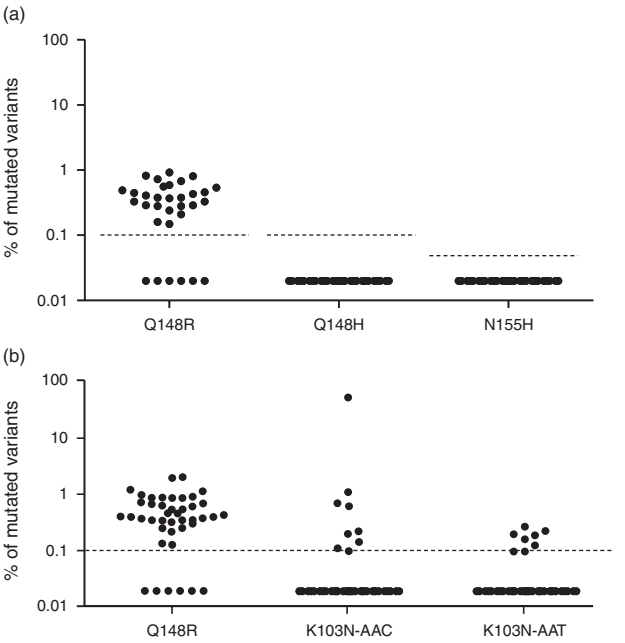

Fig. 2. Frequency of detection of integrase minority viral variants. (a) Antiretroviral-experienced patients. The AS-PCR assays designed for Q148R, Q148H and N155H mutations were performed in plasma samples from 32 highly antiretroviral-experienced patients, naive of integrase inhibitors. The AS-PCR assay limit of detection of each mutation is indicated by a dashed line. (b) Antiretroviral-naive patients. The AS-PCR assays designed for the Q148R mutation in integrase gene and the K103N mutation in reverse transcriptase gene were performed in plasma samples from 47 antiretroviral-naive patients. The AS-PCR assay limit of detection of each mutation is indicated by a dashed line. Note, values below the limit of detection were coded as 0.02%. AS-PCR, allele-specific real-time PCR.

HIV-1 minority variants in antiretroviral-naive patients

Detection of Q148R minority variants in antiretroviral-naive patients

Genetic evolution of the integrase gene during long-term ART with reverse transcriptase inhibitors was previously reported [19–21], we assessed plasma samples from 51 antiretroviral-naive patients for the presence of Q148R minority variants. Median HIV-1 viral load and CD4 T-cell count were 4.57 $\log_{10}$ copies/ml (IQR = 3.92–5.22) and 436 cells/μl (IQR = 270–535), respectively. AS-PCR assay was successful for 42 samples (82%). Q148R minority variants were detected in 36 of 42 samples (86%) at a low-level median frequency of 0.46%

(IQR = 0.35–0.87) (Fig. 2b). The proportion of patients with Q148R minority variants was similar to that observed in highly pretreated patients.

Detection of K103N minority variants in antiretroviral-naive patients

The presence of K103N minority variants in antiretroviral-naive patients has been well described in previous studies [11–15] and was demonstrated to increase the risk of virological failure to NNRTI-based therapy. Thus, to allow the comparison between the proportion of patients exhibiting Q148R, K103N minority species or both, we performed AS-PCR assays, detecting AAC and AAT changes at position 103 of reverse transcriptase in the same population of antiretroviral-naive patients.

The AS-PCR K103N assay was successful for 47 of 51 samples (92%). K103N minority variants were found in 12 patients (26%) with a median frequency of 0.21% (IQR = 0.12–0.89) and 0.18% (IQR = 0.11–0.22) for AAC and AAT changes, respectively (Fig. 2b). Among the 12 patients, five (11%) exhibited both AAC and AAT-mutated virus at position 103. The proportion of patients harboring K103N minority variants was significantly lower as compared with Q148R minority variants ($P < 0.0001$). In one patient, the K103N mutation was detected by direct sequencing and found at high proportion (50%) by AS-PCR assay, suggesting that he had been infected by a drug-resistant virus.

Discussion

We developed AS-PCR assays for mutations associated with integrase inhibitors resistance (Q148H, Q148R and N155H). The mismatches primers design and the validation process resulted in the ability of the different AS-PCR assays to detect minority variants as low as 0.10% of the plasma viral population. Unexpectedly, we found a high proportion of pretreated and antiretroviral-naive patients exhibiting minority Q148R-mutated variants at baseline. Conversely, no Q148H and no N155H minority variants were detected in heavily pretreated patients initiating salvage RAL-based therapy. Of note, the use, on the same samples, of an alternative selective Q148R primer with different mismatches generated similar results (data not shown).

Preliminary data assessing the presence of minority RAL-resistant variants using another sensitive technology, parallel allele-specific sequencing (PASS), also showed the absence of N155H minority variants among 32 samples tested [22]. Minority Q148H and Q148R-mutated variants were found in only one patient at very low frequency [22]. In the single small study [23] using ultra-deep pyrosequencing, the only minority variant found at baseline prior to RAL treatment also exhibited the Q148R mutation in one out of seven highly pretreated patients. In the present study, we found a higher proportion of patients (81% of pretreated patients) who displayed Q148R minority variants. This may be related to the methods used to detect minor viral populations. Thus, the prevalence of minority variants exhibiting reverse transcriptase mutations such as K103N or M184V in antiretroviral-naive patients differed according to the technique used. K103N minority variants were found in 4–21% of patients when tested by AS-PCR assays [12,15,24,25], but only in 0.40% of patients ($n = 258$) in the study using ultra-deep sequencing [14] and in none of the 12 patients tested with the PASS assay [26]. Of note, in our study, almost all patients were infected with HIV-1 subtype B, thus we cannot exclude that the prevalence of Q148R minority variants can be subtype dependent, as no data are available with HIV non-B subtypes.

Previous studies [19,20] had suggested that the frequency of integrase mutations at some specific codons might differ between untreated patients and reverse transcriptase inhibitors (RTIs)-treated patients. In addition, Buzón *et al.* [21] assessed evolution of integrase gene over time in patients receiving a RTI-containing regimen and found that the amino acid substitution rate within integrase gene was 0.06% per year during long-term ART without, however, any change in RAL phenotypic susceptibility. These findings argue for tight interactions between reverse transcriptase and integrase enzymes, which can lead to a potential coevolution of some of their mutations. Thus, we assessed the prevalence of Q148R minority variants in antiretroviral-naive patients in order to investigate the potential impact of the pretreatment by RTI in our study population of antiretroviral-experienced patients. A similar high proportion of antiretroviral-naive patients exhibited Q148R minority variants, indicating that the high prevalence of Q148R minority variants found in antiretroviral-experienced patients is not a consequence from a history of long-term RTI-containing therapies.

The present study reports for the first time the frequency of detection of integrase-mutated minority variants in antiretroviral-naive patients. In contrast, data are available on the prevalence of K103N minority variants in antiretroviral-naive patients [12,13,24,25]. We were interested in comparing the prevalence of K103N and Q148R-mutated variants obtained with AS-PCR assays in the same patients' population. In our study, K103N minority variants were detected in 26% of antiretroviral-naive patients, similar results that were found in previous studies [15,24,25]. Thus, Q148R minority variants were more frequently detected than K103N minority variants. Several studies [11–14] reported that the preexistence of

NNRTI-resistant variants in naive patients increased the risk of subsequent virological failure. In the present study, 24 of the 26 patients who exhibited Q148R minority variants at baseline achieved undetectable plasma HIV-1 RNA levels. For 20 of 26 patients, undetectable viral load was obtained before month 3 of RAL treatment. In contrast, four patients displayed a delayed virological response occurring between weeks 18 and 36, a mechanism known to be associated with an increased risk of resistance selection, as previously described with NRTI and NNRTI drug classes [25]. In addition, two patients with Q148R minority variants at baseline experienced a virological failure, with plasma virus developing RAL resistance using the Q148R pathway in both cases. However, we cannot establish that Q148R variants present at the time of virological failure originated from Q148R variants present at baseline. The expansion of Q148R preexisting minority variants was suggested in the study of Ceccherini *et al.* [23]. Conversely, in another preliminary study [22], none of the failure samples ($n = 14$) displayed minority variants with primary RAL resistance mutations at baseline.

The high proportion of patients exhibiting Q148R minority species at baseline and the few patients who experienced virological failure prevented from performing reliable statistical analyses to assess the impact of Q148R minority species at baseline on the subsequent virological response to IN-based regimen

In summary, the present study demonstrates that a high proportion of pretreated and antiretroviral-naive patients exhibit minority Q148R-mutated variants but no Q148H and no N155H minority variants. Although the presence of low-abundant Q148R variants was not consistently associated with virological failure, their impact on long-term viral suppression needs to be further investigated in larger series of patients to demonstrate the clinical relevance of the presence of minority Q148R-mutated variants.

Presented in part at the 16th Conference of Retroviruses and Opportunistic Infections (CROI); 8–11 February 2009; Montreal, Canada.

References

1. Steigbigel RT, Cooper DA, Kumar PN, Eron JE, Schechter M, Markowitz M, *et al.* **Raltegravir with optimized background therapy for resistant HIV-1 infection.** *N Engl J Med* 2008; **359**:339–354.
2. Markowitz M, Nguyen BY, Gotuzzo E, Mendo F, Ratanasuwan W, Kovacs C, *et al.* **Rapid and durable antiretroviral effect of the HIV-1 integrase inhibitor raltegravir as part of combination therapy in treatment-naive patients with HIV-1 infection: results of a 48-week controlled study.** *J Acquir Immune Defic Syndr* 2007; **46**:125–133.
3. Cooper DA, Steigbigel RT, Gatell JM, Rockstroh JK, Katlama C, Yeni P, *et al.* **Subgroup and resistance analyses of raltegravir for resistant HIV-1 infection.** *N Engl J Med* 2008; **359**:355–365.
4. Hazuda DJ, Miller MD, Nguyen BY, Zhao J. **Resistance to the HIV-integrase inhibitor raltegravir: analysis of protocol 005, a phase 2 study in patients with triple-class resistant HIV-1 infection** [abstract #8]. *XVI International HIV Drug Resistance Workshop*; 12–16 June 2007; Los Barbados, West Indies; 2007.
5. Fransen S, Gupta S, Frantzell A, Petropoulos C, Huang W. **HIV-1 mutations at positions 143, 148, and 155 of integrase define different genetic barriers to raltegravir resistance in vivo** [abstract #69]. *16th Conference on Retroviruses and Opportunistic Infections*; 8–11 February 2009; Montreal, Quebec, Canada; 2009.
6. Coffin JM. **HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy.** *Science* 1995; **267**:483–489.
7. Günthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD. **Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples.** *AIDS Res Hum Retroviruses* 1998; **14**:869–876.
8. Charpentier C, Dwyer DE, Mammano F, Lecossier D, Clavel F, Hance AJ. **Role of minority populations of human immunodeficiency virus type 1 in the evolution of viral resistance to protease inhibitors.** *J Virol* 2004; **78**:4234–4247.
9. Lecossier D, Shulman NS, Morand-Joubert L, Shafer RW, Joly V, Zolopa AR, *et al.* **Detection of minority populations of HIV-1 expressing the K103N resistance mutation in patients failing nevirapine.** *J Acquir Immune Defic Syndr* 2005; **38**: 37–42.
10. Roquebert B, Malet I, Wirden M, Tubiana R, Valantin MA, Simon A, *et al.* **Role of HIV-1 minority populations on resistance mutational pattern evolution and susceptibility to protease inhibitors.** *AIDS* 2006; **20**:287–289.
11. Paredes R, Lalama C, Ribaudo H, Schackman B, Shikuma C, Meyer W, *et al.* **Presence of minor populations of Y181C mutants detected by allele-specific PCR and risk of efavirenz failure in treatment-naïve patients: results of an ACTG 5095 case-cohort study** [abstract #83]. *15th Conference on Retroviruses and Opportunistic Infections*; 3–6 February 2008; Boston, Massachusetts, USA; 2008.
12. Johnson JA, Li JF, Wei X, Lipscomb J, Irlbeck D, Craig C, *et al.* **Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naïve populations and associate with reduced treatment efficacy.** *PLoS Med* 2008; **5**:e158.
13. Metzner KJ, Giulieri SG, Knoepfel SA, Rauch P, Burgisser P, Yerly S, *et al.* **Minority quasispecies of drug-resistant HIV-1 that lead to early therapy failure in treatment-naive and -adherent patients.** *Clin Infect Dis* 2009; **48**:239–247.
14. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD, Baxter JD, *et al.* **Low-abundance drug-resistant viral variants in chronically hiv-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes.** *J Infect Dis* 2009; **199**:693–701.
15. Peuchant O, Thiébaut R, Capdepont S, Lavignolle-Aurillac V, Neau D, Morlat P. **Transmission of HIV-1 minority-resistant variants and response to first-line antiretroviral therapy.** *AIDS* 2008; **22**:1417–1423.
16. Huang MM, Arnheim N, Goodman MF. **Extension of base mispairs by Taq DNA polymerase: implications for single nucleotide discrimination in PCR.** *Nucleic Acids Res* 1992; **20**:4567–4573.
17. Herring BL, Cunningham AL, Dwyer DE. **Potential drug resistance polymorphisms in the integrase gene of HIV type 1 subtype A.** *AIDS Res Hum Retroviruses* 2004; **20**:1010–1014.
18. Charpentier C, Karmochkine M, Laureillard D, Tisserand P, Bélec L, Weiss L, *et al.* **Drug resistance profiles of HIV integrase gene in patients failing raltegravir-salvage therapy.** *HIV Med* 2008; **9**:765–770.
19. Ceccherini-Silberstein F, Malet I, Fabeni L, Svicher V, Gori C, Dimonte S, *et al.* **Specific mutations related to resistance to HIV-1 integrase inhibitors are associated with reverse transcriptase mutations in HAART-treated patients** [abstract #4]. *XVI International HIV Drug Resistance Workshop*; 12–16 June 2007; Los Barbados, West Indies; 2007.

20. Suzuki H, Fujino M, Matsuda M, Yen H, Iwantani Y, Sugiura W. **Effects of protease and reverse transcriptase inhibitor-resistance mutations on integrase polymorphism in multidrug resistance cases** [abstract #2]. *XVI International HIV Drug Resistance Workshop*; 12–16 June 2007; Los Barbados, West Indies; 2007.
21. Buzón MJ, Marfil S, Puertas MC, Garcia E, Clotet B, Ruiz L, *et al*. **Raltegravir susceptibility and fitness progression of HIV type-1 integrase in patients on long-term antiretroviral therapy.** *Antivir Ther* 2008; **13**:881–893.
22. Liu J, Miller M, Danovich R, Vandergrift N, Cai F, Hicks C, *et al*. **Detection of low-frequency mutations associated with drug resistance to raltegravir before ART** [abstract #685]. *16th Conference on Retroviruses and Opportunistic Infections*; 8–11 February 2009; Montreal, Quebec, Canada; 2009.
23. Ceccherini-Silberstein F, Armenia D, D'Arrigo R, Vandenbroucke I, Van Baelen K, Van Marck H, *et al*. **Baseline variability of HIV-1 integrase in multiexperienced patients treated with raltegravir: a refined analysis by pyrosequencing** [abstract #682]. *16th Conference on Retroviruses and Opportunistic Infections*; 8–11 February 2009; Montreal, Quebec, Canada; 2009.
24. Metzner K, Rauch P, Walter H, Boesecke C, Zollner B, Jessen H, *et al*. **Detection of minor populations of drug-resistant HIV-1 in acute seroconverters.** *AIDS* 2005; **19**:1819–1825.
25. Metzner KJ, Allers K, Rauch P, Harrer T. **Rapid selection of drug-resistant HIV-1 during the first months of suppressive ART I n treatment-naive patients.** *AIDS* 2007; **21**:703–711.
26. Cai F, Chen H, Hicks CB, Bartlett JA, Zhu J, Gao F. **Detection of minor drug-resistant populations by parallel allele-specific sequencing**. *Nat Methods* 2007; **4**:123–125.