# Evolution patterns of raltegravir-resistant mutations after integrase inhibitor interruption

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## Abstract

The objective of this study was to address the evolution of human immunodeficiency virus type 1 (HIV-1) mutations resistant to the integrase inhibitor raltegravir after drug interruption. Thirteen HIV-1 infected patients undergoing virological failure due to the selection of raltegravir-resistant variants, who had interrupted raltegravir treatment, were enrolled. For all patients, the virological failure was associated with the selection of variants, with mutations conferring resistance to all of the drugs present in their regimens. Patients were prospectively monitored at baseline (raltegravir interruption) and every 4–24 weeks for clinical, virological and immunological parameters, including HIV-1 viraemia, $CD4^+$ T-cell counts, and sequence analysis of the HIV-1 integrase sequence. Reversion to the wild-type HIV-1 integrase sequence genotype was observed between 4 and 36 weeks after raltegravir withdrawal in eight out of the 13 patients. Reversion was not observed in three patients. In two patients, reversion was partial at week 24 from raltegravir interruption. These results highlight that in eight out of 13 patients under treatment with raltegravir and experiencing a virological failure, HIV-1 variants harbouring mutations associated with raltegravir resistance become undetectable after drug interruption within a few weeks (in some cases, very rapidly). This occurs under different therapy regimens and in patients receiving 3TC mono-therapy. In the other patients, complete reversion of the integrase sequence is not observed, and either primary or secondary resistance mutations are fixed in the replication competent viral population *in vivo* also for long time, suggesting that other factors may influence this dynamic process.

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## Introduction

Raltegravir, the prototype of a new class of anti-retroviral compounds (integrase inhibitors; INI), has been demonstrated to be an effective drug in the treatment of either naïve or experienced human immunodeficiency virus type 1 (HIV-1) infected subjects [1–4]. As for other classes of anti-retroviral drugs, the selection of variants carrying drug-resistance-associated mutations in the HIV-1 integrase sequence has been described in patients not responding to raltegravir including regimens [1]. Moreover, in subjects maintaining raltegravir in their regimen despite the selection of resistance mutations, a detectable increase in HIV-1 resistance levels to raltegravir driven by the continuous evolution of the viral integrase sequence has been documented [5–7]. Finally, comparative analyses of the geno-phenotypic features of resistant variants selected during raltegravir treatment *in vivo* have indicated that the replication capacity (RC) of resistant clones is compromised in INI-resistant HIV-1 variants [8–12].

In the past two decades, studies of different classes of anti-retroviral compounds have demonstrated that viral reversion from highly resistant to fully susceptible wild-type viral variants is generally observed within a variable time in cell-free plasma virus after interruption of the compounds [13,14]. It is presently believed that the lower relative fitness of resistant variants allows the reversion to wild-type archival provirus sequences in the absence of drug-selection pressure. Of note, secondary (or compensatory) and rarely primary mutations (such as the 103N in the reverse

transcriptase HIV-1 sequence) not associated with reduction of viral RC [15] are maintained in some cases after drug interruption, thus remaining as genotypic scars in the replicating virus. These footprints of previous failing regimens may be useful from a diagnostic point of view in either naïve or treatment-exposed patients, facilitating the determination of the most reliable historical GSS score [16]. Recently, several studies have evaluated the genotypic and phenotypic patterns of raltegravir-resistant variants selected *in vivo*, elucidating the role of drug resistance and RC in patients not responding to regimens including raltegravir [8–12]. However, data on the genotypic monitoring of the evolution of raltegravir-associated resistance mutations after drug interruption are still limited. Due to the importance of increasing the therapeutic options for HIV-1 infected subjects, these results could be central to the understanding of the biology of raltegravir-resistant variants and to the diagnostic management of patients treated with raltegravir.

## Patients and Methods

This research was conducted in accordance with the Declaration of Helsinki and national and institutional standards and was approved by the San Raffaele Ethical Committee.

### Genotypic analyses, virological and immunological evaluation

Thirteen patients (age, 49 ± 11 years; 11 male, 2 female), who underwent virological failure due to the selection of HIV-1 (subtype B) resistant variants, and who had interrupted raltegravir treatment, were enrolled in the present study. All patients had a long history of treatment (on average 16 years of documented antiretroviral therapy), with an average duration of HIV-1 infection of 19 years.

Patients were prospectively monitored at baseline (time of raltegravir interruption) and every 4–32 weeks for clinical, virological and immunological parameters, including HIV-1 viraemia (Versant HIV-qRNA 3.0 Assay; Siemens Healthcare Diagnostics, Deerfield, IL, USA), and $CD4^+$ T-cell counts (Fig. 1 and Table 1).

Genotypic analyses of the reverse transcriptase and protease sequences and of the *env* gene to evaluate the presence of mutations associated with drug resistance or changes in viral tropism, were also performed at the beginning of the raltegravir-including regimen, at virological rebound and during maintenance of raltegravir despite failure [5,9]. After raltegravir interruption (the baseline point for this study) pol and env genotypic analyses were performed at multiple time-points (every 4–40 weeks) (for Methods see Ref. [5 and 9]).

### Amplification of HIV-1 integrase sequence

Viral RNA was purified using the QIAmp viral RNA mini kit (Qiagen, Valencia, CA, USA). Only one sample at each time point was processed, and clinical samples and all amplification steps were carried out using a limiting dilution strategy to minimize artificial recombination events. The integrase region spanning codons 1–288 was targeted, using the following nested-RT-PCR using primers Int1F, 5′- CAT GGG TAC CAG CAC ACA CAA AGG-3′ and Int1R, 5′-CCA TGT TCT AAT CCT CAT CCT GTC -3′ for the first PCR round, while primers Int2F 5′-GGA ATT GGA GGA AAT GAA CAA GTA GAT -3′ and Int2R 5′GCC ACA CAA TCA TCA CCT GCC ATC-3′ were used in the second PCR round. The first nested-RT-PCR reaction was performed in 50 $\mu$L using the SuperScriptTM III Platinum High-Fidelity One-Step qRT-PCR System (Invitrogen, Carlsbad, CA, USA) with the following thermal profile: 30 min at 50°C and 10 min at 95°C for 1 cycle, 1 min at 95°C, 1 min at 52°C and 1 min and 10 s at 72°C for 50 cycles followed by 10 min at 72°C. The nested PCR reaction was performed in 100 $\mu$L using the PCR SuperMix High Fidelity (Invitrogen) with the same thermal profile. Direct sequencing was performed using an ABI PRISM 3100 Genetic Analyzer® (Applied Biosystem, Foster City, CA, USA). Resistance to raltegravir was evaluated according to the Stanford database report and published *ex vivo* phenotypic data [8–12]. As evaluated by serial dilutions of TA-cloned (Invitrogen) reference (NL4-3 derived) amplicones spiked in plasma samples obtained from HIV-1-negative patients, this assay was demonstrated to have an analytical sensitivity of about 240 copies with subtype B variants [9,17]. The electropherogram was analysed manually by a trained virologist to guarantee that resistant variants present in a proportion higher than 5–10% in the viral population could be identified.

## Results and Discussion

In the present study, 13 HIV-1 infected patients not responding to a raltegravir-containing regimen were enrolled. Virological and immunological parameters as well as the therapeutic regimens are shown in Fig. 1 and Table 1. For all patients, virological failure was associated with selection of variants with mutations conferring resistance to all of the drugs present in their regimens (data not shown). In particular, failure of response to raltegravir was associated with the selection of variants displaying different combinations of primary and secondary mutations after 4–56 weeks (Table 1). In many of them, especially in those where failure was associated with signature mutations other than the