Appearance of a Single Amino Acid Insertion at Position 33 in HIV Type 1 Protease Under a Lopinavir-Containing Regimen, Associated with Reduced Protease Inhibitor Susceptibility

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Abstract

HIV drug resistance is a multifactorial phenomenon and constitutes a major concern as it results in therapy failure. The aim of this study was to assess the impact of an amino acid insertion identified at position 33 of the protease gene, derived from samples from three patients under lopinavir therapy, on viral fitness and protease inhibitor (PI) resistance. Successive samples were available from one of the patients for genotypic and phenotypic testing in order to investigate the role of this insertion. The patient had been pretreated with various antiretroviral drugs and showed poor virological response from the point of the acquisition of the mutation onward. The insertion was acquired in the context of a number of other PI mutations and was stable following acquisition. Phenotypic testing revealed reduced susceptibility to various PIs and a reduction of the replicative capacity (RC) of the virus. In the presence of the insertion alone, a decrease of the RC was observed, which seemed to be compensated by the presence of other mutations. The L33ins might have a potential role in PI resistance pathways but further investigation in a larger number of clinical samples is required in order to elucidate this resistance mechanism.

Introduction

Development of HIV-1 drug resistance constitutes one of the main reasons for highly active antiretroviral therapy (HAART) failure. Drug resistance is associated with specific amino acid replacements accumulated in the protease (PR), reverse transcriptase (RT), integrase (IN), and gp41 regions selected by regimens including protease (PIs), reverse transcriptase (RTIs), integrase (INIs), and fusion inhibitors (FIs), respectively. Several mutational patterns have been described, including point mutations as well as deletions and insertions, conferring resistance to different drugs.1

Insertion mutations occur less frequently than single amino acid substitutions.2–4 The 69 insertion complex in the RT region, consisting of a substitution at codon 69 and an insertion of two or more amino acids, has been found to be associated with resistance to almost all the currently available nucleoside reverse transcriptase inhibitors (NRTIs).1,2,4–9 The 69 insertion complex has been observed at a frequency of 0.5% to 2% in individuals failing therapy.4,6,7

A survey of HIV-1 sequences from over 24,000 patients revealed that insertions in protease were present in 24 individuals3 and in another study the prevalence of insertions in protease has been found to be 10-fold lower than in the RT region among the same patient population.6 Most of the observed insertions (19 out of 24) consisted of one to five amino acids between codons 35 and 38. In vitro susceptibility assays of the viral isolates with insertions in protease revealed that reduced susceptibility to PIs was mainly associated with previously reported single amino acid mutations, thus
suggesting that the reported insertions have a minimal effect on resistance.\textsuperscript{3} A study by Stürmer \textit{et al.} reported a six-base pair insertion at codon 36 in a PI-naïve patient with no \textit{in vitro} resistance to PIs and a good \textit{in vivo} response to indinavir, suggesting that this insertion was unable to cause resistance by itself.\textsuperscript{10} In contrast, an insertion at codon 19, selected during \textit{in vivo} PI exposure before the appearance of M46I, T74P, and I84V, was found to augment resistance to all PIs.\textsuperscript{11} An insertion at codon 32 has been previously genotypically characterized in a heavily pretreated patient with multiple other PI mutations and poor virological response.\textsuperscript{12}

The aim of this study was to assess the impact of the L33 insertion on viral fitness and to investigate the different levels of PI resistance potentially arising by its emergence. Furthermore, we investigated the structural effect of the L33ins on the complex of the protease and lopinavir (LPV), under which pressure the insertion was shown to emerge, and we estimated the frequency of the appearance of L33ins in patients failing lopinavir/ritonavir (LPV/r)-containing regimens.

\section*{Materials and Methods}

\textbf{Patient samples}

In the Athens, Leuven, and Portuguese resistance databases (9000 patients in total), L33L-L was found in three patients. Successive entries per patient were analyzed, and from patient 1, seven samples were available, from 1998 to 2004 at specific time points (1998, 2001, 2002, two samples in the year 2003, and two samples in the year 2004). Similarly, four samples were available for patient 2 (2002, 2003, and two samples in the year 2007) and five samples for patient 3 (in the years 2003, 2004, 2005, 2006, and 2008). Viral RNA levels were determined using the Versant HIV-1 RNA 3.0 assay (bDNA) (Diagnósticos Division, Bayer Corp., Tarrytown, NY) or previous releases.

\textbf{Genotyping}

Genotypic resistance testing was performed using the TrueGene HIV-1 Genotyping Test, according to the manufacturer’s recommendations (Bayer Healthcare Diagnostics) or ViroSeq (Celera Diagnostics/Abbott Laboratories). The HIVdb program (Stanford) v 6.0.6 was used for resistance interpretation. Subtyping was performed using the Rega subtyping tool v2.\textsuperscript{13} The sequences of the viruses described in the study were submitted in GenBank (sequence accession numbers: HQ834788, HQ834789, HQ993086, HQ993087, HQ834790, HQ834791, HQ993088, HQ993089, HQ834792, HQ834793, HQ993090, HQ993091, and HQ993092).

\textbf{Susceptibility testing for patient strains and mutant clones}

The susceptibility of mutant viruses to various PIs was determined as described previously, with some modifications on the cloning strategy.\textsuperscript{14} Briefly, a 1.5-kb fragment encoding the C-terminus of gag, all 99 amino acids of protease, and 312 amino acids at the N-terminus of RT was amplified from two samples derived from patient 1 with reverse transcriptase polymerase chain reaction (RT-PCR) primers that incorporated Aprl and AgeI restriction sites, and then cloned into a replication-defective variant of the pNL4-3 vector. This vector contains Aprl and AgeI sites in the gag and RT coding regions, respectively, as well as a firefly luciferase gene in the nef region and a frameshift in the cem gene. For construction of molecular clones, mutations in the protease gene (L33ins) were introduced via site-directed mutagenesis into the same pNL4-3 vector in order to assess the effect of the insertion alone. Recombinant pseudotyped virus stock was generated by cotransfection of individual pNL4-3 constructs containing only the protease gene from patient isolates or specific mutations in the protease gene into HEK-293 cells along with a plasmid encoding the VSV-G envelope protein, in the presence or absence of PIs. The pseudotyped virus was used to infect fresh HEK-293 cells for determination of replication capacity (RC) and drug susceptibility using a single-cycle assay. Fifty percent inhibitory concentration (IC\textsubscript{50}) values were determined by nonlinear regression analysis with GraphPad Prism 4.0 software. Drug resistance levels of the mutants were calculated by dividing the IC\textsubscript{50} value of the mutant by the wild-type virus IC\textsubscript{50} value, and were expressed as fold-changes (FC). The RC for each mutant was calculated by comparing the luciferase activity generated by the mutant virus to that generated by wild-type virus in the absence of drug.

Phenotypic resistance testing was performed on the remaining samples derived from patient 1 using the PhenoSense HIV Drug Resistance Assay at Monogram Clinical Reference Laboratory (Monogram, CA) and replicative capacity testing was performed using the PhenoSense HIV Drug Resistance Assay. For one sample derived from patient 2, phenotyping was available from VIRCO (VIRCO BVBA, Mechelen, Belgium).

\section*{Results}

\textbf{Prevalence of L33L-L}

The Athens, Leuven, and Portuguese resistance database together contains data from about 9000 patients. The L33ins was detected in three patients, all when under an LPV/r-containing regimen, the first line for one of them. The overall prevalence of the L33ins was 0.03%. A total of 717 patients had a genotype under an LPV/r-containing regimen and thus 0.4% of them showed this insertion. For all three patients, the insertion mutation remained stable (>4 years) even after LPV/r was withdrawn.

\textbf{Genotyping}

Patient 1 was found to harbor a subtype B virus. The patient CD4 and viral load history is depicted in Fig. 1. IDV treatment lasted from August 1997 until February 1999. Genotypic resistance testing revealed a total of 11 amino acid changes including mutations associated with PI resistance scoring intermediate resistance to LPV and high level resistance to other PIs by HIVdb (Table 1). Following cessation of PI treatment, genotypic tests showed that the virus retained only the L10V, I13V, and L63P mutations. The virus reverted back to the previous genotypic profile 16 months after initiation of LPV treatment. Specifically, the L10V mutation evolved to the L10F and it acquired a single amino acid insertion of leucine (TTA) at position 33 (flap region) (Fig. 2). All mutations, including the insertion, were maintained in all consecutive samples after September 2002 (Table 1). The patient’s viral load remained high following the acquisition of the insertion.
The patient strains with the insertion mutation for the other two patients belonged to subtype G (patient 2) and a unique recombinant form, mainly consisting of subtype F in the region analyzed (patient 3). For patient 2, the insertion appeared following 16 months of LPV/r treatment. The patient had previously received a nelfinavir (NFV)-containing regimen and had a genotype that scored low level or intermediate resistance for LPV/r by HIVdb and Regav8.0.2, respectively (Table 1). 89M is wild type and 10I, 36I, 63P, and 69K are common polymorphisms in subtype G. The next genotype during LPV/r treatment displayed additional amino acid changes and was shown to be highly resistant to LPV by both algorithms, even when not taking into account the insertion. From then on the insertion was stable; it disappeared briefly in the absence of PIs when the original wild-type strain reemerged, but appeared again as soon as a saquinavir/ritonavir (SQV/r)-containing regimen was installed, under which 48V, 62V, 73S, and 89V were added. The patient never had an undetectable viral load.

Patient 3 developed the insertion almost 2 years after starting a first line HAART with LPV/r, 1 year after already scoring resistant according to both HIVdb and Regav8.0.2.

![FIG. 1. Viral load, CD4 cell count measurements, and treatment history of patient 1.](image)

Table 1. **Resistance-Associated Mutations Observed over Time in the Study Population**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sampling date</th>
<th>Resistance mutations in PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3TC + d4T + IDV</td>
<td>20/2/2001</td>
<td>L10V, I13V, L63P</td>
</tr>
</tbody>
</table>

3TC, lamivudine; d4T, stavudine; IDV, indinavir; ddI, didanosine; EFV, efavirenz; ABV, abacavir; HU, hydroxyurea; LPV/r, lopinavir/ritonavir; TDF, tenofovir; AZT, zidovudine; NFV, nelfinavir; ATV/r, atazanavir/ritonavir; SQV, saquinavir; ABC, abacavir; FTC, emtricitabine.
At the time the insertion was observed, other resistance mutations were also present (Table 1). Under a subsequent SQV/  
r-containing regimen, G48Q, F53L, and L90M were added, but the insertion remained stable. This patient also never reached an undetectable viral load.

**Phenotyping**

Results on PI susceptibility of the viral clones containing the protease sequence derived from two samples of patient 1 showed an increased IC₅₀ for amprenavir (APV), NFV, and LPV compared to wild type (Table 2). Specifically, for the sample collected in the year 1998, an IC₅₀ fold change of 33 LPV compared to wild type (Table 2). The replicative capacity values for the two samples were also reduced to 12% and 5.9%, respectively. No significant change in the susceptibility of any of the PIs tested following the emergence of the insertion the strain showed resistance to most of the PIs, including LPV (IC₅₀ fold change > 92.7 by Antivirogram).

Similarly, phenotypic tests by Phenosense revealed reduced susceptibility to APV, LPV, indinavir (IDV), and ritonavir (RTV) for the two samples collected in the years 2002 and 2004 (Table 2). The replicative capacity values for the two samples were also reduced to 12% and 5.9%, respectively. No significant change in the susceptibility of any of the PIs tested was observed for the sample collected in the year 2001 (while the patient was not under PI therapy). The RC value of the same sample was reported at 66%.

Limited phenotypic data are also available for patient 2. Following the emergence of the insertion the strain showed resistance to most of the PIs, including LPV (IC₅₀ fold change > 92.7 by Antivirogram).

The effect of the insertion on PI susceptibility at position 33 was also assessed in the context of the pNLC4-3 genetic background. Results revealed that the insertion conferred no resistance to any of the PIs and could possibly increase susceptibility to some of them (LPV: 0.80-fold, RTV: 0.46-fold, AV: 0.26-fold, IND: 0.43-fold).
The protease sequence derived from patient 1 samples at two time points, relative to pNL4-3 wild type. The replicative capacity of all strains from patient 1 harboring the insertion was shown to be diminished compared to wild type. Although a decreasing trend in the replicative capacity of the isolates was observed, the insertion mutant was the dominant population. Previous reports on the effect of protease insertions on viral replication are conflicting. Studies have reported a compensating role of insertions for the replication of the virus, whereas others report both a protective and a reductive effect, suggesting that the effect on the replication on the virus might be insertion specific. Furthermore, in a recent study conducted by Virco, insertions at site 33 were detected at a prevalence of 0.1% and the amino acids observed were E, Q, V, and L in order of frequency. In all patients, the L33 insertions were accompanied by a group of other PI mutations. This suggests that the incidence of the insertion could be favored under specific circumstances such as a high number of preexisting mutations and might be acting as a stabilizing agent, contributing to the prevalence of the specific viral population. The replicative capacity caused by the in vitro insertion could explain the fact that the insertion has never been reported to occur on its own.

In patient 1, the virus harboring L33ins and other protease mutations showed a reduction in the susceptibility to APV and LPV. The increase in the APV IC₅₀ values could be explained as a result of the presence of the M46I and I84V mutations, which are associated with APV resistance. In all patients, the L33 insertions were accompanied by a group of other PI mutations. This suggests that the incidence of the insertion could be favored under specific circumstances such as a high number of preexisting mutations and might be acting as a stabilizing agent, contributing to the prevalence of the specific viral population. It is true that phenotyping is often requested only when many mutations complicate genotypic interpretation. The dramatic decrease in the replicative capacity caused by the insertion in vitro could explain the fact that the insertion has never been reported to occur on its own.

In patient 1, the virus harboring L33ins and other protease mutations showed a reduction in the susceptibility to APV and LPV. The increase in the APV IC₅₀ values could be explained as a result of the presence of the M46I and I84V mutations, which are associated with APV resistance. However, the high IC₅₀ values observed for LPV in the samples collected in the years 2002 and 2004 cannot be explained by the specific genotypic profile, as the particular amino acid changes in the patient are not predictive of high level resistance to LPV, suggesting a potential role of the insertion in the resistance level to the drug. Furthermore, data from another study report various levels of PI resistance in patients harboring L33 insertions as part of a cluster of mutations with the L33Q shown to cause a 5-fold increase in the IC₅₀ of darunavir (DRV). The replicative capacity of all strains from patient 1 harboring the insertion was shown to be diminished compared to wild type. Although a decreasing trend in the replicative capacity of the isolates was observed, the insertion mutant was the dominant population. Previous reports on the effect of protease insertions on viral replication are conflicting. Studies have reported a compensating role of insertions for the replication of the virus, whereas others report both a protective and a reductive effect, suggesting that the effect on the replication on the virus might be insertion specific.

Furthermore, the insertion was also detected in two patients infected with different subtypes (G and URF mainly F) and at least in subtype G was shown to confer resistance to a
number of PIs. This might suggest a potential role of the insertion in resistance in other HIV subtypes.

As mentioned above, the presence of the insertion alone was shown to interfere with viral replication in vitro. Similar results have been reported previously for other protease insertions such as the insertion at position 35, which was not shown to confer PI resistance but affected the replicative capacity of the virus.17

There have been a number of reports on insertions occurring in the protease, allowing the identification of some common parameters such as the following: (1) they arise in heavily pretreated patients,3,12,16,22 (2) the patients show a poor virological response,12,16 (3) upon acquiring the insertion the genotypes remain stable,3,12,16 and (4) they occur as a part of a cluster of mutations.3,12,16,17,20,22 The above suggest that protease insertions could be part of a specific resistance mechanism facilitating the evolution of multi-PI-resistant strains. Furthermore, a higher frequency of occurrence of such insertions is observed between codons 30 and 40,3,22 indicating that this might be an insertion-prone area with a potential role in resistance mechanisms.

In conclusion, we report a single amino acid insertion at position 33 of protease (L33L-L) in three patients infected with three different subtypes, selected under treatment with LPV/r. The insertion was found to have an effect on viral fitness on its own and in combination with other PI mutations it was shown to contribute to reduced susceptibility to NVP, APV, and LPV. These data indicate an alternative to a single amino acid replacement pathway for developing PI resistance.

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Author Disclosure Statement

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