Original Article

Deletion, insertion and stop codon mutations in *vif* genes of HIV-1 infecting slow progressor patients

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Abstract

Background: Variable progression towards AIDS has been described and has been related to viral and host factors. Around 10% of the HIV-1 infected patients are slow progressors (SP), not presenting with AIDS disease signs even after more than 10 years of infection. Viral gene defects have been associated with the disease progression but more studies are still needed.
Methodology: The sequence of *vif* and *nef* were analyzed for HIV-1 infecting 14 SP and 46 normal progressors (NP) patients.
Results: Co-circulation of a strain carrying *vif* deleted gene with the wild type strain was detected in an SP patient with more than 10 years of infection. Other mutations (insertion in aa 63 in one strain, two premature stop codons in another one) were found in viruses infecting two other patients. Except for the SP8 strain, which exhibited a premature stop codon in *nef*, no gross deletions or insertions were observed in *nef* genes of both NPs and SPs strains analyzed.
Conclusions: Different kind of mutation: deletion, insertion and stop codon, were detected in 3/14 samples from SP, with co-circulation of a 195 bp *vif* deletion virus with a wild type in one of these patients. Although *vif* defects do not seem to be a frequent feature in SPs, this study illustrates the importance of analysing this gene, in addition to the multiple factors associated with the long-term non progression to AIDS.

Key Words: HIV, APOBEC, long term progressor, controller.

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Introduction

The temporal progression to AIDS shows a high degree of variability. In some cases rapid progression may occur after only three years, leading to death, usually in patients perinatally infected. In other uncommon cases, patients do not develop disease and exhibit a long survival rate after several years of infection [1]. This later group of patients is known as slow progressors or long-term non progressors (SP or LTNP) and represent around 10% of the total HIV-1 infected patients [2]. The mechanisms leading to a long survival in SP are not fully understood. At least two factors have been proposed: those related to the host immune response and susceptibility, and those related to the viral replication capacity. In the latter, viral attenuation might be partially responsible for the slow viral replication capacity [3].

The accessory genes in HIV-1 are responsible for many regulatory functions in the viral cycle to circumvent antiviral intrinsic immunity and to promote T lymphocyte activation. Among them, the lentiviral protein Nef creates an optimal environment for lentivirus replication by activating resting T cells [4]. HIV-1 and SIV also encode Vif (viral infectivity factor), an accessory protein that counteracts the antiviral activity of the APOBEC3 family. APOBEC3 enzymes belong to the superfamily of cytidine deaminases [5] that induce a high rate of dC to dU mutation in the first minus strand of cDNA, causing hypermutation and degradation throughout the HIV-1 genome [6,7]. The deaminases A3G, A3F, and A3B have potent antiviral activity, with the first two being expressed in cells that are susceptible to HIV-1 infection; Vif binds to APOBEC3G/3F but not A3B and induces its degradation through polyubiquitination [8,9].

Both viral and host factors participate in HIV-1 disease progression [1,10]. Three rates of progression towards AIDS disease have been described: rapid progressors, who develop disease within a period of less than three years after initial infection with the virus; normal progressors (NP), whose immune systems remain intact during the early part of infection but show gradual deterioration over a period

of 10-15 years; and long-term non-progressors (SP or LTNP), who remaine healthy, with normal CD4+ and CD8+ T-cell counts and low but detectable plasma viremia, or below the detection limit. The latter group represents 8-10% of the total HIV-1 infected population [11]. Studies of the viruses infecting these patients have shown alterations in one or more HIV-1 accessory genes, which seem to correlate with disease progression; particularly, several deletions and polymorphisms have been described in *nef* genes [12,13]. However, although the importance of *vif* gene is quite clear at present, few polymorphisms have been described associated with a retarded progression to AIDS. The aim of this study was to analyze two accessory genes in SP HIV-1 infected patients, *nef* and *vif*, in order to identify eventual mutations that could be associated with the slow progression in these patients.

Materials and Methods

Population groups

A total of 14 patients were classified as SP based on previously described criteria [14,15]: 1) more than three years of diagnosis of HIV-1 infection (range 3 to 19 years); 2) in this period of time the viral load was maintained in low levels (below detectable levels or below 10,000 copies/ml); and 3) CD4 cell count remained constant without a significant drop below 500 cell/µl. All SP were naïve, except for SP9 who started treatment one year before the time of blood collection; naïve NP (n = 47), with at least three years of diagnosis (range 3 to 11 years) were used as controls. Viral load for these patients was significantly higher (geometric mean of viral load 65,000 copies/ml). Some of the NP started HAART shortly after the sampling for this study.

Vif *and* nef *sequence analysis*

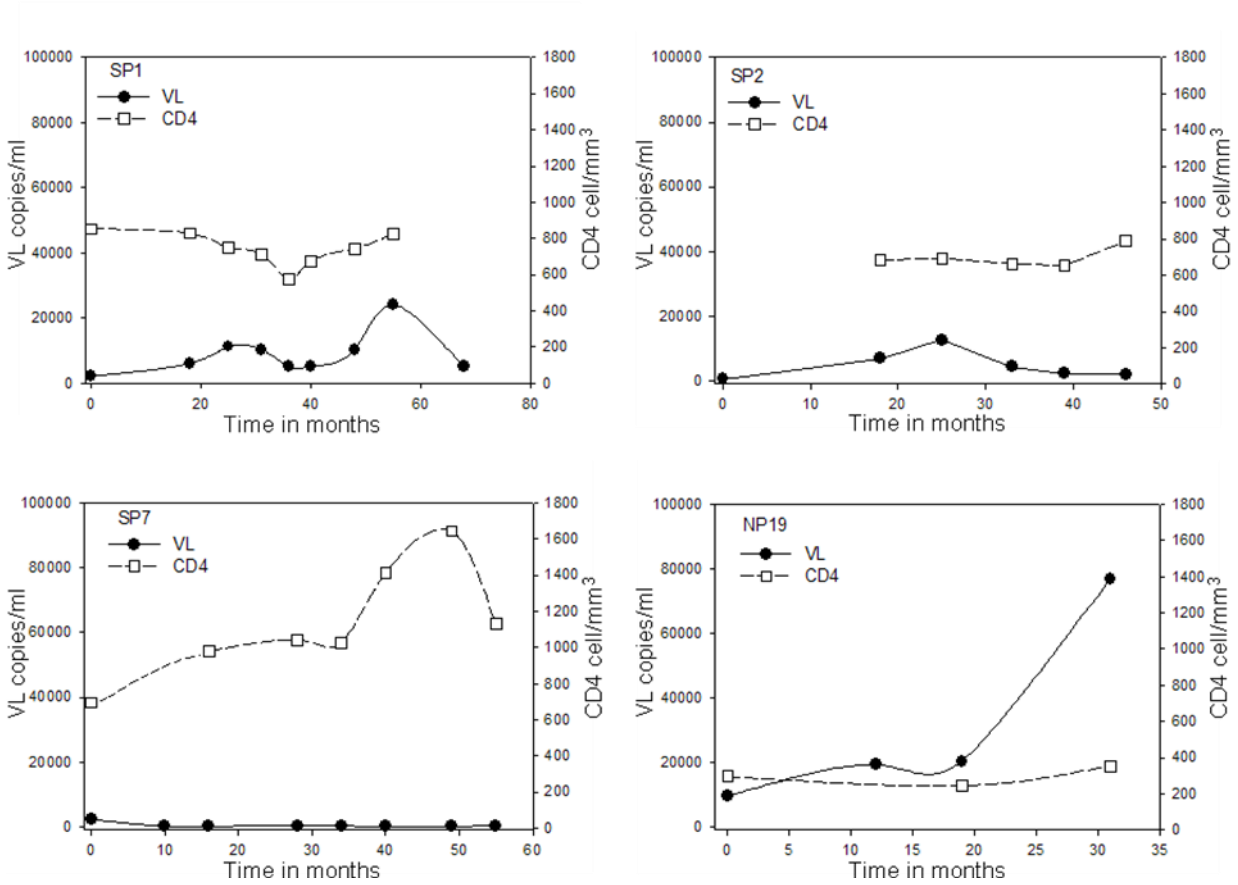
Viral RNA was extracted from plasma of HIV-1 infected patients using commercial kits (QIAamp® Viral RNA Kit, QIAGEN, Germany). The C-terminal region of Integrase gene and 181 of the 192 aa sequence of *vif* (678 bp) was amplified by RT-nested PCR with primers Vif-1S 5´-tcgggtttattacagggac-3´ and Vif-1AS 5´-cttattatggcttccactcc-3´ as external primers and a second round with Vif-2S 5´-tggaaaggaccagcaaagc-3´ and Vif-2AS 5´-attcattgtatggctccctct-3´ under the following conditions: 2 min at 95°C, then 95°C 30 sec, 55°C 30 sec, 72°C 1 min for 39 cycles with a final extension of 7 min at 72°C, for both rounds. *Nef* gene (919 bp) was amplified by RT-nested PCR with primers Nef 1S (5´-gtagctgaagggacagatagggttat-3´), Nef 1AS (5´-gcactcaaggcaagctttattgaggc-3´) for first round and Nef 2S (5´-acatacctagaagaataagacagg-3´) and Nef 2AS (5´-gtccccagcggaaagtcccttgta-3´) for the second round [16]. Vif PCR product from SP#1 was also cloned by AT overhang (PCR®2.1TOPO®, Invitrogen Corporation USA). Both clones and amplicons were sequenced (Macrogen Service Center, Korea). The electropherograms of the sequences were inspected visually for the presence of multiple nucleotides in a single position (quasispecies). Nucleotide alignments and phylogenetic analysis were performed using DNAMAN Version 5.2.2. (Lynnon Bio Soft, Canada).

Results

PCR products from *vif* genes (almost complete gene) were obtained from 14 SP and 46 NP patients. *nef* gene (complete gene) was amplified for 7 SP and 35 NP. Representative follow-up of three SP and one NP is shown in Figure 1. While CD4 levels remained generally over 600 cells /ml for SP, they were found around 400 or lower for NP. For example, for NP19, viral load increased significantly during the 31-month period analyzed (Figure 1).

All the viral strains were classified as subtype B in both regions of the HIV-1 genome. The sequences from SP viral strains did not group in any cluster in any of the two viral genomic regions analyzed (data not shown). All the virus strains carried a Vif protein with the expected amino acids E^{88}, and both C^{114} and C^{133} (Figure 2), which have been shown to be critical *in vitro* for infectivity [17,18]. Tryptophan residues in positions 5, 11, 21, 38, 79, and 89, many of them essential for the selective suppression of APOBEC3G and APOBEC3F [19], were also conserved. One of the two Lys in positions 22 and 26 present in Vif has also been shown recently to be important for infectivity in non permissive cells [20]. While K^{26} was conserved among all the viral strains, K22NH was found in 3/14 viruses infecting SP, but also in 9/47 viruses infecting NP (data not shown).

The SLQYLA motif, critical for ElonginB and ElonginC binding and Vif function [21,22], was present in almost all viral strains except in SP1, SP4 and SP11 (SLQHLA, TLQYLA and TLQFLA respectively) (Figure 2). These slightly altered motifs are present, however, in some SIV strains [23] and in 3/47 NP from this study. The HCCH motif, critical for cullin5 interaction and Vif function [23,24] was also conserved in all the viral strains (Figure 2). The



multimerization motif PPLP located at the C-terminal region of Vif was also conserved among the isolates, except in one NP strain (NP13), where one nt insertion generated a change in the coding region and a premature stop codon (Figure 2). However, this patient exhibited a high viral load (125,000 copies/ml) and no apparent SP phenotype.

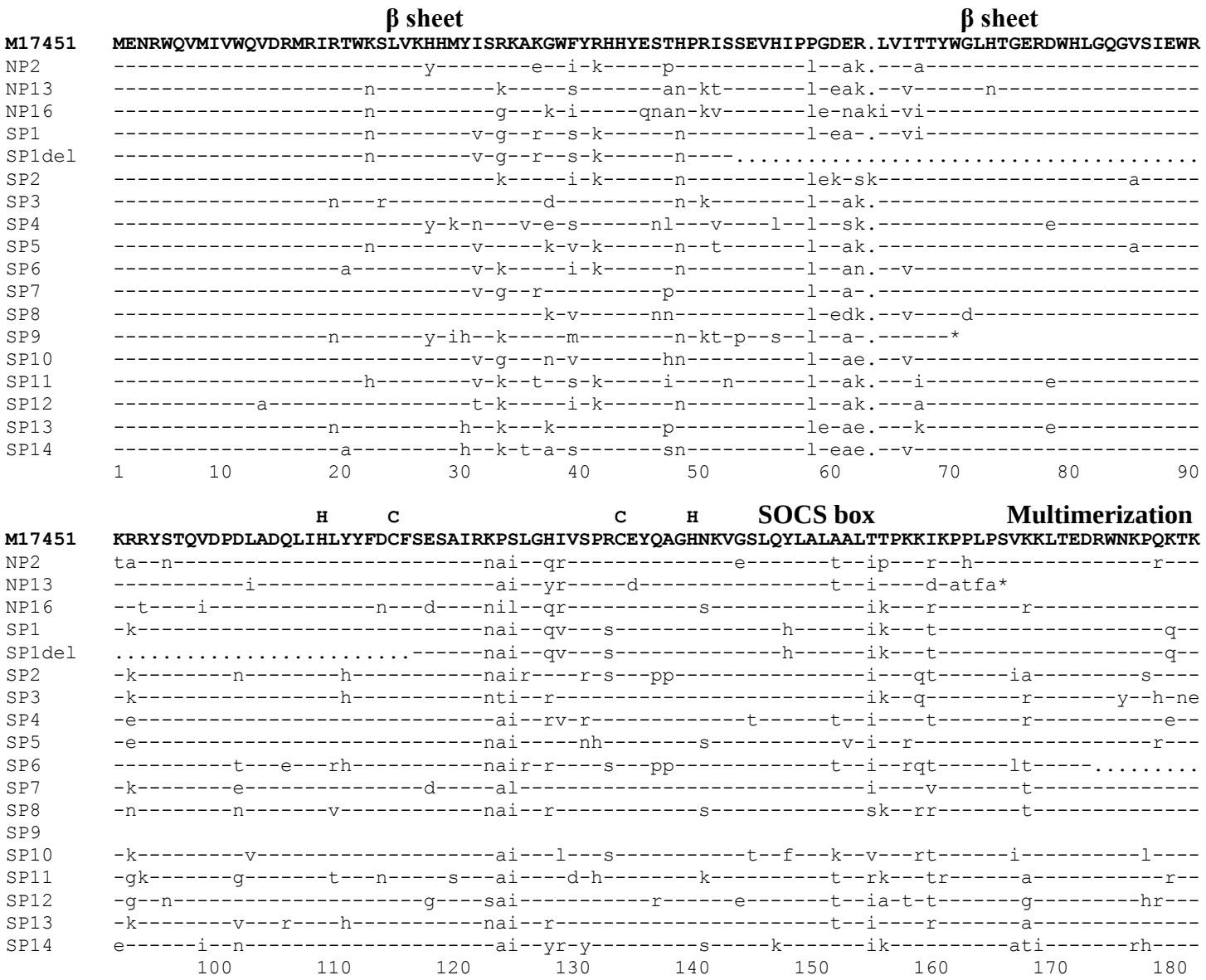
All amplicons exhibited a unique band of expected size, except for Vif PCR product from SP1, where 2 bands were observed. SP1 is a naïve masculine patient of 49 years old, with an HIV-1 positive diagnosis from 1997. Detailed analysis of the sequence electropherogram of SP1 viral strains showed an overlapped shorter sequence pattern. The sequences of the 4 clones available of the SP1 viral strain confirmed the presence of a deletion of 195 bp, corresponding to aa 54 to 117 (Figure 2). The virus exhibiting the deletion in *vif* also carried a deletion in one nucleotide in the integrase gene, which leads to a premature stop codon at amino acid position 259, interrupting the carboxy-terminal region of this enzyme, and then the non specific DNA binding motif [25,26].

Densitometric analysis showed that the intensity of the PCR band corresponding to the deleted viral strain exhibited 62% of the intensity of the non deleted viral one. A follow-up plasma sample was available for SP1 (Figure 1). One year after the first sampling analyzed in this study, the patient experienced a significant increase in viral load. This elevation of viral load coincided with a significant reduction in circulation of the deleted Vif variant, detected as the absence of a minor band in the PCR product of a follow-up sample and a reduction in the intensity of the sequence of the deleted gene by visual inspection of the electropherogram (data not shown).

SP2 exhibited stable levels of CD4 and low viral load (under 10000 copies/ml) for a period of at least 46 months, and was diagnosed more than six years ago (Figure 1). Vif protein of the HIV-1 infecting strain harbored an insertion in position 63 (Figure 2).

The viral strains were also analyzed for the presence of hypermutagenesis by using the algorithm Hypermut 2.0 (www.hiv.lanl.gov). The concatenated *vif-nef* sequences and the individual sequences were analyzed when available, in order to increase the size

Figure 2: *Vif* (aa 1 to 180) amino acid sequences of HIV-1 infecting SP.



Some NP variants are shown for comparison, and an HIV-1 subtype B sequence was used as reference (GenBank accession number M17451). Motifs essential for proper *vif* activity are shown[34]. **β sheet:** domains essential for β sheet formation. **SOCS box**: motif required for *vif* phosphorylation and Elongin BC and Cullin5 Binding. **H-5-C-18-C-5-H:** Motif essential for Cullin5 interaction and *vif* function. **Multimerization:** domain required for *vif* multimerization and subsequent function.

of the sequence analyzed. In addition, for patient SP9, a sequence of POL region (1490 bp) was analyzed and hypermutation was detected in this region, but not in the concatenated *vif-nef* sequence. SP9 maintained an undetectable viral load for at least 19 years, with stable CD4 counts. This patient initiated treatment, despite maintaining a relatively low viral load (2,000 copies/ml), the year before this study. Several drug resistance mutations were found after one year of treatment. The *vif* sequence of SP9 viral strain contained two premature stop codons at positions 70 and 174 (Figure 2). Coding codons were not observed as quasispecies in any of these two positions. The sequence of SP4 viral strain also showed hypermutation in the concatenated *vif-nef* region and in the *nef* gene but not in the *vif* gene alone. No hypermutation was detected in any other viral sequence.

R132S substitution has been described in viral strains replicating a lower viral load. This mutation has also been shown *in vitro* to reduce the viral replication [2,27]. This substitution was present in viruses infecting 17/46 NP and with a similar same frequency in 3/9 SP. In addition, two strains from NP harbored a K in this position and one I respectively. In NP, the geometric mean of viral load in NP harboring viruses with R^{132} or K^{132} of Vif was 61,400 copies/ml, significantly higher than the mean viral load in NP carrying viruses with an S in this position (21,900 copies/ml, Student t test p = 0.006). In the same way, the number of NP with a viral load of 55,000 copies/ml [28] or lower was higher among NP infected with virus with a S^{132} in Vif compared with NP with viruses carrying an R or K in this position (14/23 vs. 3/23, Chi square test p = 0.002).

Except for SP8 strain, where a premature stop codon was observed in *nef* in aa 6, no gross deletion or insertion was observed in any of the other genes amplified, from SP or NP patients: some small insertions and deletions were observed in SP viral strain, which were also present in NP viral strain (data not shown).

Discussion

Sequence analysis of the *vif* gene from patient SP1 showed the co-circulation of a strain carrying a deleted *vif* gene, around one third (38%) of the total viral population, with the wild type virus. HIV-1 viruses with deletion and/or mutation in a specific position of *vif* gene are generally less infective than wild types. Indeed, feline immunodeficiency viruses carrying deleted *vif* gene have been described as an attenuated virus in kitten and adult felines [29] and deletion associated with *vif* gene from simian immunodeficiency viruses has shown to generate more attenuated virus, compared to other accessory genes [30]. The degree of attenuation depends on the site and extension of the deletion. Vif protein with in-frame deletion of amino acids 23 to 43 could still interact with APOBEC3G [31], but may promote its degradation with lower efficiency than the wild type. Due to the extensive deletion observed in the variant of SP1 Vif, even if this truncated protein may be synthesized, we can predict that this accessory variant protein is not active. The region deleted in the HIV-1 strain from SP1 comprises amino acids 57 to 71, a region which has been shown critical for APOBEC3G binding [32], and part of the zinc-binding region [33] (Figure 2). As most of the motifs previously described, the deletion in SP1 covers amino acids that have been shown by He *et al.* [31] to be important for APOBEC3G degradation. Based on these results, the circulation in blood of this viral variant might be due to complementation between the wild type Vif and the deleted one. Interference and trans complementation of defective HIV-1 and wild type viruses has been described *in vitro* [34,35]. A delay in infection and production has also been observed *in vitro* with HIV-1 with defective Vif [9,36]. Quasispecies in proviral *vif* genes, showing the coexistence of functional and deleted or truncated *vif* genes, have also been described [37] and this coexistence might be related to attenuation. Although the relative frequency of functional Vif versus inactive ones was not associated with non progression in the mentioned study, this phenomenon might be contributing at least in some, to a delayed progression of AIDS [38]. The presence of this truncated *vif* in a virus from a SP harboring a complete *nef* gene suggests that this mutation might be contributing by itself to the non progression phenotype.

The analysis of a follow-up plasma sample, available for SP1, showed the reduction in circulation of the deleted Vif variant with a simultaneous increase in viral load. Then, a significant increase in viral load for this patient was associated with a reduction in the circulation of the variant with a defective *vif*. Since only a limited volume of plasma sample was available for these patients, genetic susceptibility to HIV-1 infection, like CCR5 polymorphism, could not be determined in this group of patients. This factor might have contributed in some of them to the slow progression of disease.

However, the temporal correlation between the presence of the mutant strain, with the significant reduction in viral load, suggests that mutant *vif* present in quasispecies contributed to the SP phenotype in this patient. During the natural course of its infection, the wild type virus seemed to take advantage over the variant, which is consistent with the exclusion principle, where, in a competition for the niche, one species always will eliminate the other [39]. In this case, the wild type virus seemed to take advantage over the *vif* deleted variant. In addition, it cannot be ruled out that the premature stop codon in the integrase of the mutant strain might also contribute significantly to the SP phenotype of this viral quasispecies.

A two amino acid insertion close to *vif* position 63, the aa where an insertion was found in SP2, has been described to reduce drastically the functionality of *vif* gene [10]. Functionality studies of Vif protein with an insertion in this position might bring some clues about the contribution of this particular mutation to the slow progression phenotype observed in this patient.

The presence of HIV-1 viral strains carrying Vif with suboptimal APOBEC3G suppressive activity favors the appearance of drug resistance mutation [40]. In this study, hypermutation was detected in only two viruses infecting SP patients. Hypermutation is not equally distributed along the HIV-1 genome [41,42]. In addition, it is important to note that except for SP9, for which proviral DNA was analyzed, in all other patients the sequence was obtained from viral RNA. This might have also limited the detection of hypermutation in the viral genomes, since the proviral compartment has been shown to exhibit more frequent hypermutation [41]. The premature stop codon 70 observed in strain SP9 has been described previously in a virus infecting an SP, which also harbored a deleted *nef* gene [14]. In a study of 127 sequences of HIV-1, evidence of hypermutagenesis was found in 15 strains. The viral load was significantly lower in the patients harbouring these strains [43]. Premature stop codons in *vif*, particularly in codon 70, were significantly found in these strains. However, the authors suggest that the premature stop codons are located in the target TGG motif of APOBEC3G, and then it is difficult to elucidate if these premature codons are the cause or the consequence of hypermutagenesis.

R132S substitution has been described in viral strains replicating at a lower rate. This mutation has also been shown *in vitro* to reduce viral replication [2,27]. Patients infected with viruses carrying a Vif protein with S132 exhibited a lower mean viral load than those infected with the wild type virus. Although this comparison is hampered by the variability in time of infection of the studied naïve patients, Vif with S^{132} seems to be associated with viruses circulating at lower viral load also in patients progressing classically to AIDS. Indeed, the mean viral load among NP carrying the R132S substitution was very similar to the one described for SP by Hassaïne *et al*. [2].

In conclusion, three out of the 14 *vif* genes circulating in SP harbored mutations (deletion, insertion or premature stop codons) that might be contributing to the slow disease progression in these patients. In one of these viral strains with mutated *vif*, hypermutation could be detected in the HIV-1 genome. No significant alterations were found in all *nef* genes, but one of the studied patients. Other studies, involving a similar modest number of SP, have not identified *vif* mutations as responsible for the SP phenotype [44,45]. The results from this study suggest that Vif defects do not seem to be a frequent feature in SPs. However, Vif phenotypic alterations may be contributing, at least temporarily during the natural course of infection, to a reduction in HIV-1 replication *in vivo* and then to a SP phenotype. These results warrant the importance of adding *vif* gene analysis to the multiple factors associated with the long-term non progression to AIDS.

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Sequence data

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are EU432502-EU432511, FJ641183-FJ641187 and FJ659410-FJ659504.

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