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Sequence Note

Natural Polymorphisms of HIV Type 2 *pol* Sequences from Drug-Naive Individuals

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ABSTRACT

Until today, the susceptibility of human immunodeficiency virus type 2 (HIV-2) to protease and nucleosidic reverse-transcriptase inhibitors (PI and NRTI, respectively) has not been clearly documented. In this report we studied HIV-2 proviral sequences ($n = 30$) from drug-naive patients. Our results revealed that several amino acid positions in the protease and reverse transcriptase coding sequence harbored residues that have been associated with drug resistance in HIV-1-infected patients. In particular, the M46I substitution in the protease was detected in 90% of the sequences analyzed, which, together with the other substitutions identified, may indicate a reduced susceptibility of HIV-2-infected drug-naive patients to PI. Furthermore, interpretation of genotypic data with four available algorithms, developed for interpretation of HIV-1 sequence data, suggested nonoverlapping profiles of drug resistance.

THE IMPRESSIVE DEGREE OF GENETIC VARIABILITY displayed by the human immunodeficiency virus (HIV) accounts for the continuous generation of molecular variants, and endows this infectious agent with the capacity to rapidly overcome environmental selective pressures, such as those imposed by the host immune system or antiretroviral (ARV) therapy.[1]

Both HIV-1 and HIV-2 cause AIDS, but while HIV-1 has spread all over the world, HIV-2 infections remain, somehow, restricted to West Africa. A noteworthy exception is Portugal, where HIV-2 still accounts for approximately 4% of the total number of AIDS cases (information available at http://www.insarj.pt).

Over the past decade, the implementation of highly effective combination therapy with ARV drugs has greatly reduced the morbidity and mortality among HIV-infected individuals. The main therapeutic drugs used to restrain viral multiplication target two essential viral enzymes, both encoded by the *pol* gene: the protease (PR) and the reverse transcriptase (RT). However, in those cases in which only partial suppression of viral replication is achieved, emergence and selection of drug-resistant viruses, which tend to accumulate over time, seems to be a common event.[2]

Numerous studies focused on HIV drug resistance have correlated critical amino acid substitutions in Pol with resistance to PR inhibitors (PI), nucleoside/nucleotide RT inhibitors (NRTI), and non-NRTI (NNRTI).[3,4] Most of the current knowledge on HIV drug resistance is, however, based on data obtained for HIV-1 subtype B. Comparatively less is known about HIV-2, for which a commercially available method for quantification of viral RNA in the plasma is still lacking, and large-scale clinical trials using ARV drugs have yet to be conducted.

Despite the low sequence identity between the HIV-1 and HIV-2 genomes, *pol* genes from these viruses share 50% identity in the PR and 60% in the RT sequences, and both enzymes retain the same overall functionality.[5–7] This finding led to the suggestion that at least some of the drugs effective against HIV-1 could also be useful to manage HIV-2 infections.[8] Accordingly, and despite the fact that knowledge about treatment of HIV-2 patients is still limited (because few patients have been treated and randomized trials are lacking), it usually follows the

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same overall strategy used for HIV-1. The clinical data indicate that HIV-2 strains are sensitive to most PI and NRTI but, like HIV-1 group O, are intrinsically resistant to the currently used NNRTI.[2,9] At present, there are no available validated assays to assess HIV-2 drug-resistance (either genotypic or phenotypic) in response to ARV drug selection.[10] Moreover, to date, a limited number of studies have reported the presence of genotypic changes in the *pol* gene of HIV-2, which could be drug resistance-associated mutations.[9,11–14]

In this report we describe the natural polymorphisms in proviral *pol* genes, covering the PR and most of the RT from drug-naive HIV-2-infected patients. Our analysis enrolled 15 individuals (10 males, 5 females), 22–67 years old (mean 40.1 years), all Africans (12 of whom originated from Guinea-Bissau). The only self-reported mode of infection was heterosexual contact, and only one of them indicated having been infected in Portugal. Four of them evidenced symptoms of advanced disease.

Peripheral blood mononuclear cells were separated by density centrifugation, and the preparation of whole cell lysates was carried out as previously described.[15] Partial HIV-2 *pol* proviral sequences were amplified by nested polymerase chain reaction (PCR) using primers Pol2Fo (5′-GGAAAGAAGCCCCGCAACTTCCCCG-3′, positions 1860 to 1884 in HIV-2_{ROD}) and Pol2Ro (5′-CCTGGTGTATTTTATATGTCCACTGATTG-3′, positions 3405–3433) in the first round, and Pol2Fi (5′-CAGAGGACTTGCTGCACCTCAATTCTCTC-3′, positions 2072–2100) and Pol2Ri (5′-TTTTGGATGTCATTGACTGTCCATAYTTC-3′, positions 3145–3173) in the second round of amplification, using the puRe *Taq* Ready-to-go PCR Beads (Amersham Biosciences) as recommended by the supplier. The reaction conditions used in the first and second rounds of PCR were the same. These included an initial denaturation step at 94°C for 1 min, followed by 35 cycles of 94°C for 45 sec, 56°C for 30 sec (the hybridization temperature was lowered to 52°C in the first three cycles), and 72°C for 2 min, followed by a final extension step of 5 min at 72°C.

PCR products were directly cloned into pGEM-T Easy (Promega) and individual plasmid clones were purified and sequenced with fluorescent dye-coupled dideoxynucleotides with an ABI PRISM 377 automated sequencer (Applied Biosystems). DNA and predicted protein sequences were aligned with ClustalW (available at http://www.ebi.ac.uk/clustalw) and manually stripped for gaps. Neighbor-joining phylogenetic trees were constructed from distance matrixes calculated by the Kimura two-parameter method using the PHYLIP version 3.5 software package (through http://bioweb.pasteur.fr). Reference sequences were obtained from the Los Alamos HIV sequence database (available at http://hiv-web.lanl.gov/). HYPERMUT and SNAP software (both available at http://hiv-web.lanl.gov/) were used to detect G → A hypermutation and to calculate the rate of nonsynonymous to synonymous substitutions (*dN/dS*), respectively.

Altogether, we examined 30 nucleotide sequences that, by phylogenetic inference analysis, were ascribed to HIV-2 group A genomes (data not shown). When taken together with previous subtyping data, focused on the analysis of *env* or *nef* genes amplified from the same DNA extracts,[15,16] these results reinforce a nonrecombinant nature for the viral genomes analyzed. One of the examined sequences was found to be hypermutated due to frequent G → A replacements, particularly associated with GG and GA dinucleotides, resulting in the premature introduction of translation stop codons within the Pol coding sequence by replacement of TGG by TAG (data not shown).

Analysis of the nucleotide sequences revealed genetic diversity values (percentage) of 8.7 ± 2.4 and 9.3 ± 2.6 (mean ± standard deviation) for the PR and RT coding segments, respectively. In both regions we observed a predominance of synonymous over nonsynonymous substitutions, clearly revealed by the calculated *dN/dS* ratios (0.571 for the PR and 0.077 for the RT coding sequences), and suggestive of negative (or purifying; $dN/dS < 1$) selection.[17]

The DNA sequences obtained were inspected for natural polymorphisms, and the amino acid variability at each site was established by calculating the percentage of sequences showing a specific residue at each position. We found that 42 (44.7%) and 95 (38.1%) of the positions of the HIV-2 PR and RT, respectively, varied spontaneously (Figs. 1 and 2). Among these, positions 7, 10, 14, 40, 61, 65, 68, and 70 (in the PR, $n = 8$) and positions 4, 5, 10, 11, 28, 35, 43, 51, 83, 86, 126, 135, 162, 167, 173, 176, 180, and 228 (in the RT, $n = 18$) were considered highly polymorphic, as ≥20% of the analyzed sequences showed amino acid residues that differed from those most frequently found. The HIV-2 PR and RT amino acid sequences presented in this work are in agreement with the HIV-2 group A consensus provided in the literature (see Figs. 1 and 2), with the exception of position 167 in the RT, where the substitution of valine by an isoleucine residue is suggested (Fig. 2).

The protease presents 3 noncontiguous functionally important domains showing little amino acid variation (indicated in Fig. 1 as active site, flap region, and substrate binding domain). These correspond to domains of PR that are functionally important in HIV-1,[18] and harbor similar amino acids at most positions,[19] suggesting similar structural features for both enzymes. The PR active site and the substrate binding domain are the two best conserved regions, while the flap region (positions 47–56) accommodates a slightly higher degree of variability. Nevertheless, the patterns of natural polymorphisms between HIV-1 and HIV-2 seem to be quite different. This is exemplified by the fact that at least four of the highly polymorphic sites in the HIV-2 sequences analyzed are well conserved sequences in HIV-1 (positions 7, 14, 65, and 68). On the contrary, codon 63, which has been described as the most polymorphic position in the HIV-1 PR, was absolutely conserved in all the sequences analyzed, being occupied by a glutamate (E) residue, as previously reported.[13]

The proviral sequences obtained were inspected for detection of positions associated with HIV-1 drug resistance taking into account the International AIDS Society[4] (available at http://www.iasusa.org/resistance_mutations/resistance.pdf) and the HIVdb Drug Resistance algorithms (http://hivdb6.stanford.edu). We compared the 21 positions associated with PI resistance with the same ones in the HIV-2 sequences here described and looked for the presence of major (30N, 33I/F, 46I/L, 48V, 50L/V, 82A/F/S/T/V, 84V, and 90M) and minor (10I/V/F/R, 20I/L/M/R/T, 24I, 32I, 36I/L/V, 47A/V53L, 54A/L/M/S/T/V, 63P, 71T/V, 73A/C/S/T, 77I, and 88D/S) PI resistance mutations. The data obtained are in agreement with recent reports in the literature[13,20] as they revealed that the amino acid residues always (36I/V, 47V, 73A) or mostly found

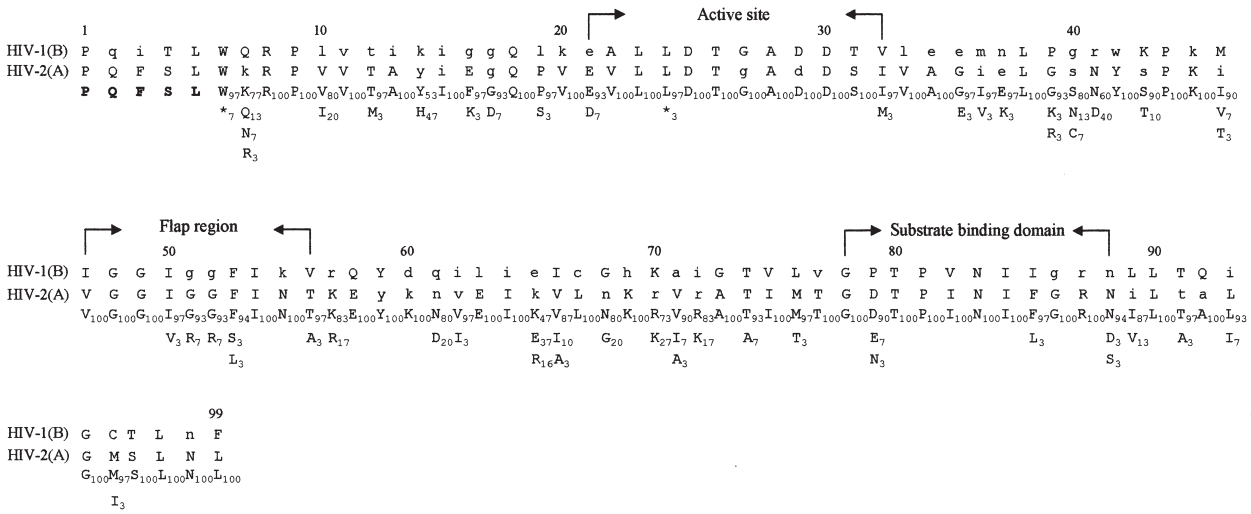

FIG. 1. Amino acid consensus derived from the multiple alignments of the deduced sequences for the HIV-2 PR. Amino acid positions are indicated above residues along the sequence. The percentage of sequences containing a given amino acid at each position is indicated as subscripts. The protease conserved domains (active site, flap region substrate binding domain) are indicated by the vertical arrows. For comparison the HIV-1 subtype B and HIV-2 group A consensus sequences [HIV-1(B) and HIV-2(A), respectively] are also indicated. The five residues in boldface at the N-terminus of the protease sequences are encoded by one of the PCR primers.

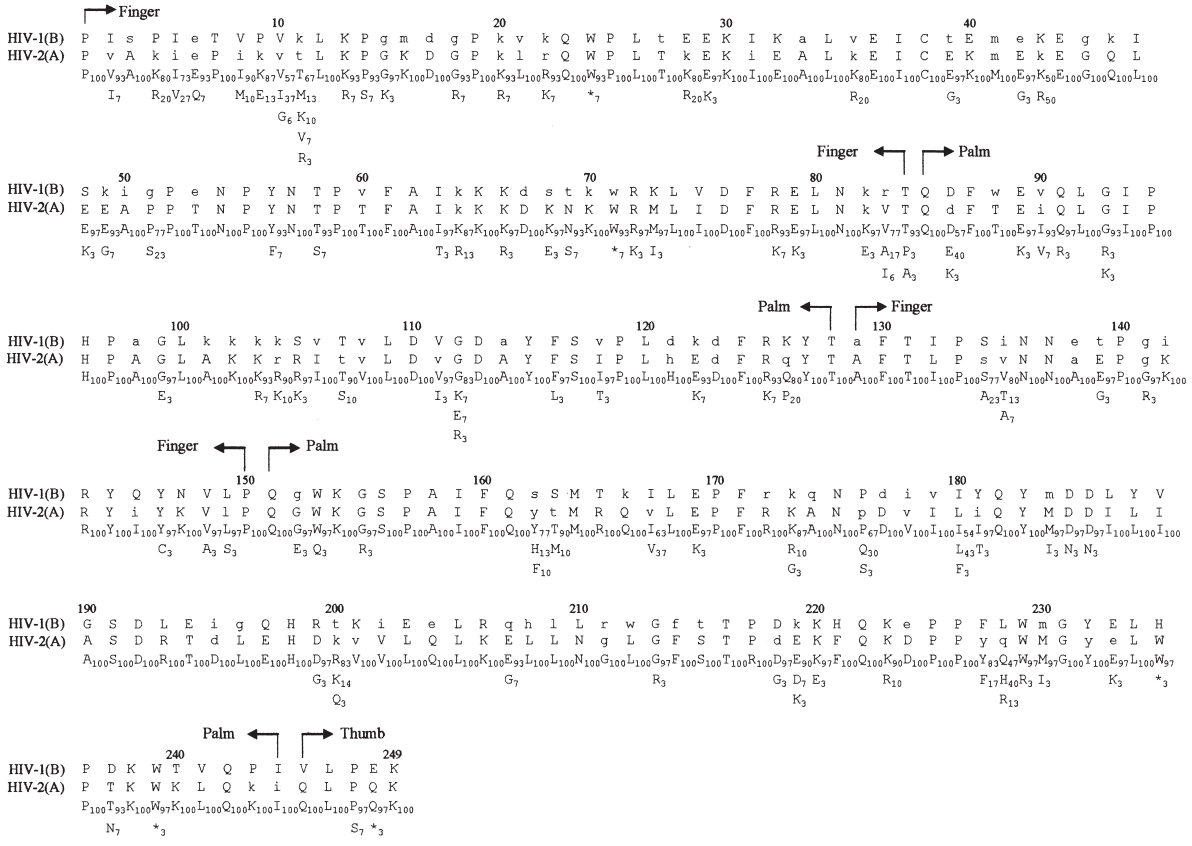

FIG. 2. Amino acid consensus derived from the multiple alignments of the deduced sequences for the HIV-2 RT. Amino acid positions are indicated above residues along the RT sequence. The percentage of sequences containing a given amino acid at each position is indicated as subscripts. The finger, palm, and thumb structural domains of the viral reverse transcriptase are indicated by the vertical arrows. For comparison, the HIV-1 subtype B and HIV-2 group A consensus sequences [HIV-1(B) and HIV-2(A), respectively] are also indicated.

(10I/V, 32I, 46I, 71I/V) at seven positions in the HIV-2 sequences correspond to those conferring (46I) or contributing (all other mutations) to PI resistance in HIV-1-infected patients. Substitutions 32I, 47V 71V, and 73A were also rarely, or never, seen in HIV-1 sequences but, on the contrary, 36I was very common in HIV-1 from drug-naive patients. In particular, substitution M46I, rarely seen in global collections of HIV-1 sequences from drug-naive individuals, associated with resistance to indinavir (IDV) or described as a minor mutation associated with resistance to ritonavir (RTV), nelfinavir (NFV), amprenavir (APV), atazanavir (ATV), and lopinavir (LPV)/RTV (http://hivdb.stanford.edu), was detected in 90% of the sequences reported here. The great majority of these harbored at least six multiple PI resistance-associated mutations in patterns featuring both the major M46I substitution and different minor ones. The most commonly observed pattern was 10I/V, 32I, 36I, 46I, 47I, 71V, and 73A, suggesting, by the HIVdb algorithm, intermediate (IR, with 10I) or high level (HLR, with 10V) resistance to APV, IR resistance to ATV, IDV, LPV, NFV, and RTV, and low level resistance (LLR) to SQV. Altogether, this combination of mutations comprises 73% of the HIV-2 sequences analyzed, followed by 10I/V, 32I, 36I, 46I, 47I, and 73A (10%, indicating IR to all PI except SQV, for which LLR is anticipated). It should, however, be mentioned that two of the sequences analyzed (with the mutation pattern 10I, 32I, 36I, 46I, 47I, 71V, and 73A) are predicted to confer IR to all PI except APV and NFV or ATV and NFV, for which HLR is expected, due to the N88D or N88S substitutions, respectively. Altogether, these observations are in agreement with previous results suggesting that HIV-2-infected drug-naive individuals may show natural reduced susceptibility to PI.[11,13,20]

The same HIV-2 nucleotide sequences were also tentatively analyzed using the Rega Institute (RegaInst, available at www.kuleuven.ac.be/rega/cev/links/) and ANRS (Agence National de Recherche sur le SIDA, available at www.hivfrenchresistance.org) algorithms, which have also been designed for the interpretation of HIV-1 genotypic resistance data. In the first case, 90% of the sequences indicate resistance to IDV and RTV. However, in the second case, sequence analysis suggested overall resistance to IDV, possible resistance to NFV in the majority of the sequences due to the widespread 36I, 46I combination.

In the RT we found that amino acid residues frequently/always localized at 11 positions (69N, 75I, 106I, 118I, 181I, 184L, 188L, 190A, 210N, 215S, and 219D), corresponded to those contributing to ARV drug resistance in HIV-1 (Fig. 2). Although some of these polymorphisms were characteristic of HIV-2 RT, other regions such as those from positions 84–101, 143–161, and 229–240 were well conserved in both HIV-1 and HIV-2 RT, and should probably be regarded in view of the design of new RT-inhibitory compounds. As expected, all the sequences analyzed evidenced at least two of the amino acid residues 181I, 188L, and 190A, all of which are major mutations associated with NNRTI resistance.

Our data also disclose the 69N/S polymorphism in all the sequences, which has been shown to confer reduced susceptibility to zidovudine (ZDV), didanoside (ddI), and stavudine (d4T). This contrasts to what is observed for HIV-1, where these residues are not usually encoded by RT sequences from drug-naive patients.[21] All the sequences analyzed evidenced the T215S substitution (typical of HIV-2 genomes) that has been described as a transitional mutation (to Y or F), conferring increased risk of virologic failure of ZDV and d4T in HIV-1-infected drug-naive adults starting therapy with these drugs.[3] However, in HIV-2, it seems that mutations at positions 151 and 219, rather than 215, may be important for development of ZDV resistance.[12] While the first of these positions was absolutely conserved, the second corresponds to a polymorphic site in the sequences analyzed. Other infrequent mutations detected, previously shown to have been selected in HIV-2 by NRTI treatment,[12,14] were 5V, 35R, and 223R. These were, however, identified in our study as natural polymorphisms in 27%, 20%, and 10% of the sequences analyzed, respectively. As reported by Colson and collaborators,[14] the amino acid residues 75I, 118I, and 219E, which potentially confer low-level resistance to some NRTI in HIV-1, were frequently found. Moreover, the 5V, 35R, and 223R mutations that emerge in HIV-2 under NRTI-selective pressure were already found at a low level (27%, 20%, and 10%, respectively) in the sequence data reported.

Interpretation of the obtained HIV-2 genotypic data with the HIVdb algorithm indicates potential IR or LLR to all the available NRTI, while the ANRS suggests potential resistance to AZT and d4T (due to the presence of a T215S substitution in all the sequences). Finally, the interpretation of the same genotypic data using the RegaInst algorithm gave somewhat less-restrictive genotypic resistance results suggesting resistance to AZT (due to the combinations of the 69N and 219E substitutions), and both ddC and ddI (due to the very frequent T69N replacement).

Despite the documented similarities in the primary sequences of the PR and RT of HIV-1 and HIV-2, some of the amino acid substitutions causing, or contributing to, resistance to different ARV drugs in HIV-1-infected patients seem to be natural polymorphisms in HIV-2. Conversely, certain natural, as well as drug-selected polymorphisms typical of HIV-2 are not, or only rarely, seen in HIV-1. Considering both situations, we still lack consistent and accurate data on the impact of the different amino acid substitutions on both viral fitness and level of drug resistance in the course of an HIV-2 infection. Furthermore, analysis of HIV-2 genotypic resistance with algorithms developed for the interpretation of HIV-1 sequence data gives rise to diversity of drug-resistance profiles. In fact, different studies seem to point toward the idea that the interpretation of HIV-2 genotypic resistance data should not be directly carried out using algorithms developed for HIV-1. For example, a previous study has suggested that the development of ZDV resistance in HIV-2 differs from that of HIV-1, and that resistance to PI appears to involve mutations in the HIV-2 PR not considered as PI-resistance mutations in HIV-1.[12] On the other hand, Witvrouw *et al*.[22] showed that despite the presence of the major 46I PR mutation (as well as several other minor ones, e.g., 10V, 32I, 36I, and 76V), HIV-2 ROD (group A) and ECHO (group B) were sensitive to IDV, RTV, and SQV, while the registered NFV and APV reduced sensitivity were not predicted from the genotypic data. These observations seem to indicate that the susceptibility of HIV-2 PR to ARV drugs is determined by amino acid substitutions other than those contributing to ARV drug resistance in HIV-1, and the same may hold true for the HIV-2 RT.

Like previous studies of genotypic resistance to ARV drugs in HIV-2, ours is limited in the amount of the data provided (number of sequences), but it documents several differences between HIV-2 group A and HIV-1 PR and RT. The identification of mutations/polymorphisms in the *pol* gene of HIV-2 drug-naive patients may be important for planning future studies that will ascertain the pathways for development of PI/RT drug resistance during ARV treatment. Indeed, as it is still rather uncertain whether the amino acid substitutions associated with drug resistance in HIV-1 and HIV-2 are the same, the algorithms for the interpretation of HIV-2 genotypic resistance should also take into account codons other than those strictly associated with ARV drug resistance in HIV-1. The way natural polymorphisms of the HIV-2 PR and RT impact on the phenotypic susceptibility of the different viral strains to ARV drugs can be closely evaluated only by phenotypic drug susceptibility assays using HIV-2 sequences derived either directly from patient material or created by *in vitro* mutagenesis. The development of these phenotypic assays is, therefore, imperative.

SEQUENCE DATA

The nucleotide sequences reported in this study have been submitted to the EMBL/GenBank/DDBJ sequence databases under the accession numbers AJ878426–AJ878455.

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