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**ALTA PREVALENCIA DE MUTACIONES SECUNDARIAS EN AISLADOS  
VENEZOLANOS DEL VIRUS DE INMUNODEFICIENCIA HUMANA TIPO 1**

**HIGH PREVALENCE OF SECONDARY RESISTANCE MUTATIONS IN  
VENEZUELAN HIV-1 ISOLATES**

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**Short Title: Genotypic resistance in HIV-1 Venezuelan isolates**

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## **RESUMEN**

Se estudiaron pacientes seropositivos para el virus de inmunodeficiencia humana tipo 1 (VIH-1) con y sin tratamiento, con el fin de determinar el polimorfismo y la prevalencia de mutaciones de resistencia a la terapia antirretroviral. El material genético viral fue extraído a partir de células mononucleares de sangre periféricas (ADN) y del plasma (ARN) de 30 pacientes. Se amplificaron 2 regiones del gen Pol, Transcriptasa Reversa (TR) y Proteasa (Pr) y el gen de envoltura (Env) por medio de la técnica de PCR y se obtuvo la secuencia genómica de los productos. Todos los aislados analizados pertenecieron al subtipo B. No se observaron mutaciones primarias asociadas a resistencia a inhibidores de Pr pero sí un alto porcentaje (86%, 19/22) de mutaciones no asociadas con resistencia sino a restitución de la capacidad replicativa de cepas mutantes (mutaciones secundarias). Se observó la presencia de mutaciones asociadas a resistencia a inhibidores nucleósidos de la TR (INTR) en 35% (6/17) de los pacientes sometidos a tratamiento, mientras que 12% (2/17) de ellos presentaron mutaciones de resistencia a inhibidores no nucleósidos de la TR (INNTR). Interesantemente, un paciente no tratado estaba infectado con una cepa que presentaba mutaciones primarias (7,7%); este resultado sugiere que podría ser importante plantearse el estudio local de determinación de resistencia genotípica en pacientes antes del tratamiento, con miras a minimizar fallas terapéuticas. Se requieren estudios adicionales para evaluar el rol de las mutaciones secundarias en el éxito de la infección viral.

**Palabras clave:** VIH-1, Sur América, mutaciones secundarias, capacidad replicativa, recombinación.

**ABSTRACT**

The genetic variability was studied in HIV-1 from Venezuelan patients with and without treatment, in order to evaluate the presence of polymorphisms and drug resistance mutations. Proviral DNA from peripheral blood mononuclear cells or viral RNA from plasma was extracted from the blood of 30 patients. Two regions from the polymerase gene, protease (Pr) and reverse transcriptase (RT) and one genomic fragment from the envelope (Env) gene were amplified and sequenced. All HIV-1 samples analyzed were classified as subtype B, without evidence of recombination. Although no primary protease mutations were detected, a high frequency of secondary mutations (86%, 19/22), associated to restoration of viral replicative fitness, was observed in strains circulating both in treated and non-treated patients. Resistance mutations to nucleoside RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTI) were detected in 35% (6/17) and 12% (2/17) of the viruses circulating in treated patients, respectively. Resistance mutations were also present in the virus infecting one antiretroviral naïve individual (7.7%), suggesting that local screening for resistant mutation in naïve patient might be important to minimize therapy failure. Future studies are warranted to assess the role of secondary mutation in the success of viral infection.

**Keywords:** HIV-1, South America, Secondary mutations, Fitness, Recombination.

## INTRODUCTION

Around 39 million persons are infected with human immunodeficiency virus type 1 (HIV-1) worldwide, and more than 20 millions have already died from this disease (1). In Venezuela, at the end of 2003, 110000 (47000-170000) persons were estimated to be infected (1). Three groups have been described for HIV-1, M, N and O. HIV-1 group M exhibits a worldwide distribution and can be classified into 24 different genetic variants, which include at least 11 subtypes (2). Viral mutations also occur during the infection and many of them are related to the pressure exerted by the immune system and/or to the antiretroviral therapy (3).

Highly active anti-retroviral therapy (HAART), especially that including triple-drug combination, has proved to be beneficial to HIV infected patients. Effective HAART induces a sustained reduction of the plasma viral load, an increase in the CD4+ T cells counts, and also a delayed disease progression to AIDS. However, such benefits may be hampered by important risks associated with prolonged treatment, such as metabolic disturbances, pharmacological interactions, and low adherence. Such circumstances can lead to the rapid development of viral resistance (4). The susceptibility to a drug of a specific viral isolate can be tested phenotypically, by *in vitro* assay, or genotypically, by sequencing and assessment of drug resistance by evaluation through database algorithms (5). Additionally, genotypic testing offers the possibility of detecting secondary mutations, i.e., mutations which do not confer drug resistance but help in restoring the replication ability or fitness of the mutant viruses (3). The aim of this study was to pursue the molecular survey of HIV isolates circulating in Venezuela, in order to evaluate the presence of polymorphisms and drug resistance mutations.

## **MATERIALS AND METHODS**

**Patients.** A total of 30 HIV-1 positive patients (mean age: 33 years, range: 20-57, 4 women and 26 men) were included in this study, upon written consent. Seventeen patients were receiving treatment (T) and 13 were either naive or had not received treatment for at least 2 years (NT).

**PCR.** Proviral DNA and/or RNA were extracted from peripheral blood mononuclear cells (PBMC) or plasma with commercial kits (QIAamp® DNA Blood mini Kit and QIAamp® UltraSens VirusKit, QIAGEN, Germany). Two regions from the polymerase gene, reverse transcriptase (RT) and protease (Pr), and one region from envelope gene (Env) were amplified by nested PCR or RT-PCR (6). Primers used for Env amplification were: E80 (5'-ccaattccatacattattgtg-3') and E105 (5'-gcttttctacttctctgccac-3') for the first round and E110 (5'-tgtaaattggcagcttagcagaa-3') and E125 (5'-caatttctgggtcccctcctgagg-3') for the second round.

**Sequencing.** PCR fragments were sequenced using dye terminator labeling method (ABI PRISM™ Dye terminator Cycle Sequencing ready reaction Kit; Perkin Elmer; Foster; CA) with 377 DNA sequencer (Applied Biosystems, Foster, CA). Both strands of DNA were sequenced. Nucleotide alignments and phylogenetic analyses were performed using DNAMAN Version 5.2.2. (Lynnon Bio Soft, Canada). Phylogenetic trees were obtained using the Neighbor Joining Method (100 bootstrap replications). Genetic distances were evaluated with Kimura 2 parameters corrections. Nucleotide sequence data have been deposited into the GenBank database under the accession numbers AY841802-AY841853.

**Determination of genotypic resistance.** Sequences were submitted to a genotype sequence algorithm (Los Alamos Resistance Database: HIVdb: Drug Resistance Algorithm, Beta Test, [http://hivdb2.stanford.edu/asi/deployed/hiv\\_central.pl?program=hivdb](http://hivdb2.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb)). Amino acids

1 to 99 and 1 to 242 were analyzed for Pr and RT respectively. Statistical significance was assessed by the chi square test with Yate's correction, according to a computerized Epi Info program, version 5.01b (Centers for Disease Control and Prevention, Atlanta, GA, USA).

## RESULTS

Nucleic acids were extracted from 30 plasma samples and 21 PBMC to amplify products of 805, 515 y 332 nucleotides approximately, corresponding to the regions RT, Pr and Env regions, respectively. Amplification was more efficient from proviral DNA and similar for all the regions, but for none of the samples amplification was obtained for all the regions in the two compartments (data not shown).

A total of 30 sequences were available for RT region and 22 for Pr region. Sequence analysis of the polymerase region, or RT (Figure 1A) and Pr separately, showed that all HIV-1 isolates belonged to subtype B. Venezuelan isolates were not grouped either in a specific clade, or according to viral load, CD4+ T cell counts, specific mutations and/or antiretroviral treatment. Phylogenetic analysis of 22 isolates in the Env region also grouped all the Venezuelan isolates inside subtype B, therefore suggesting the absence of recombination in these isolates (Figure 1B). Phylogenetic grouping in the Env region was not supported by high bootstrap values, probably due to the short length of the region analyzed; all the isolates were grouped however inside subtype B clade and exhibited more than 78% similarity with subtype B and less than 77% with other subtypes.

Primary mutations were not observed in the Pr region, being the frequency of Pr mutations significantly lower than that recorded for RT mutations (0/22 vs. 7/30,  $p= 0.02$ ). However, the majority of the isolates exhibited secondary mutations (80%, 19/22), being the most common one in the amino acid 63 (73%), with L63P found in 56% of the

sequences analyzed (Figure 2A). Mutation in such codon was present both in treated and in naïve patients, with a similar frequency (7/8 in non treated patients vs. 10/13 in treated patients, statistically NS). Primary mutations, conferring resistance to nucleoside RT inhibitors (NRTI), were observed in 23% (7/30) of the isolates and mutations conferring resistance to non nucleoside RT inhibitors (NNRTI) in 6.7% (2/30) of the isolates (Figures 2B and 2C). The most common NRTI mutation was V118I, which confers intermediate resistance to 3TC when present with E44A/D. Six out of 17 (35%) and 2/17 (12%) of the treated patients harbored viral strains with mutations conferring resistance to NRTI and NNRTI respectively. One untreated patient (1/13, 7.7%) harbored and HIV-1 isolate with multiple NRTI mutations: this individual referred sexual contact with a partner receiving HAART.

## **DISCUSSION**

Previous studies suggest that plasma is the compartment most commonly used for assessing genotypic resistance. Although plasma better reflects the viral quasispecies circulating at a given moment in the host, and most recently selected by antiretroviral pressure, other studies have shown that the proviral DNA determination may allow for the detection of other mutations, not expressed at the moment of the testing; therefore, more suitable to test the potential of drug resistance in the strain infecting a particular host (7, 8). The choice of the most suitable compartment to assess genotypic resistance is still an open issue (9). An alternative approach would be to study several body compartments, particularly when the genotypic analysis of the viral strain does not explain the viral relapse. In general, there seems to be a good correlation between the genotypic variability observed in the proviral DNA and in the plasma viral RNA (10). In our study, most of the



genomic sequences were derived from proviral DNA, due to the higher efficiency of amplification in this compartment.

Phylogenetic analysis showed that subtype B is still highly predominant in Venezuela, as previously reported (11-15). Nevertheless, recombinant B/F (16), B/C and subtype C isolates have been described more recently (17). Recombination in retroviruses is a complex phenomenon and can occur many times in a single genome (2), which may hamper the identification of recombinant strains. Sequencing of multiple regions of HIV-1 genome is then a convenient strategy to rule out the presence of recombinant strains (18), allowing at the same time for subtype assignment and determination of genotypic resistance. The relative low diversity of HIV-1 Venezuelan isolates found in this study and in other recent surveys (11; Suarez, JA, personal communication) contrasts with the general assumption of an ascending epidemic in this country; more studies are needed to assess the actual prevalence of HIV infection in Venezuela and the eventual introduction of other HIV variants in specific epidemiological settings.

In our series, primary mutations to RT inhibitors were found mainly in patients receiving the specific drug for which the strain developed resistance, although one naive patient harbored a drug resistant isolate. Whereas the number of naive patients analyzed is low, similar results have been found by others in Venezuela (11), suggesting the circulation of resistant strains among naive patients in such a frequency that might justify the determination of genotypic resistance test before the initiation of treatment. K103N substitution, which was found in association with the NRTI V118I mutation in one treated patient, is a mutation conferring high level resistance to current NNRTI (19). This mutation has been found to persist after single dose administration of nevirapine (NVP) and might not reduce the fitness of the mutant virus (20, 21). On the other hand, no primary mutations

were observed in half of the patients (4/8) with suspicion of relapse. In these patients, viremia rebound might be due to non-adherence to antiretroviral treatment rather than virological failure.

Although rather infrequent, Y318F mutation confers resistance to delavirdine (DLV) and low level resistance to NVP (21). Amino acids 318 and 333 were not analyzed in this study. Nevertheless, it must be emphasized that some commercial genotyping assays do not analyze these amino acids either.

The absence of primary mutations in the Pr region observed in our study is consistent with what has been already found by others in Venezuela (11), and might be influenced by the low number of samples analyzed for Pr mutations. On the other hand, a relatively high frequency of secondary mutations, not necessarily associated with HAART, was found in this protein, particularly L63P mutation. Such mutations may contribute to a rapid evolution of the resistance strain selected under Pr inhibitor pressure (22). The role of these secondary mutations in the replicative fitness of wild-type viruses deserves further clarification.

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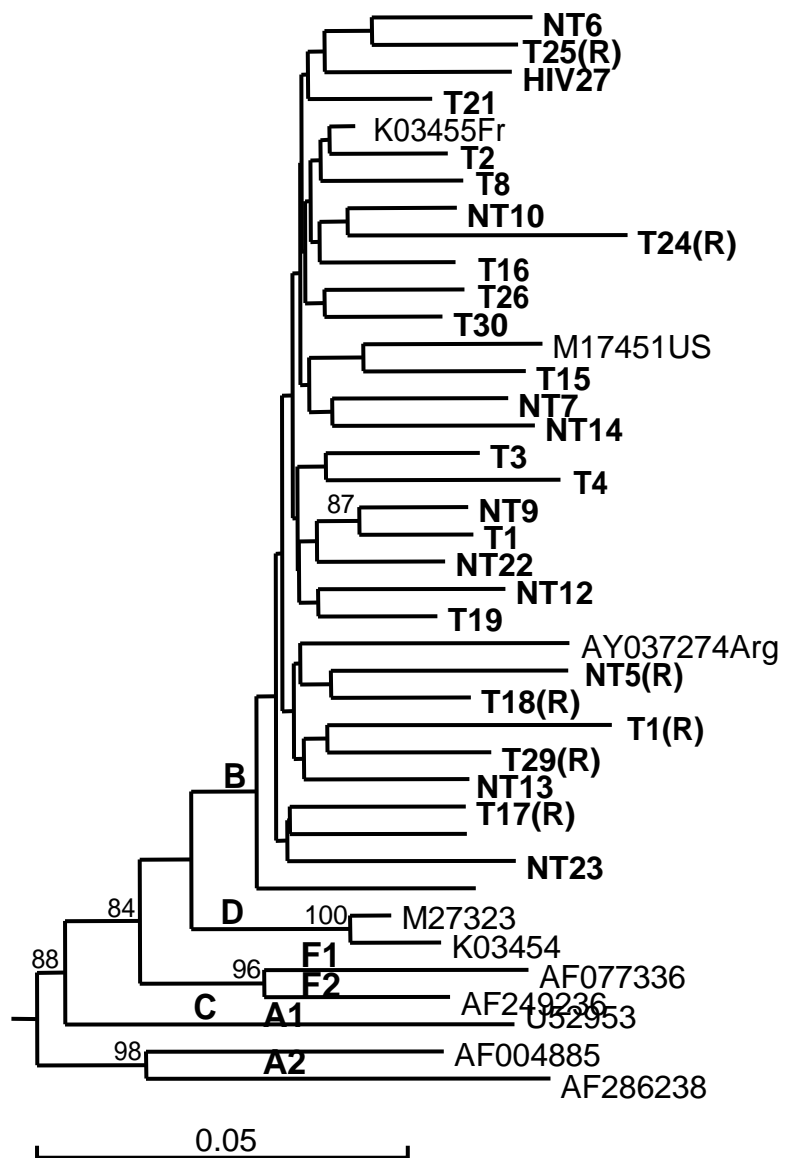
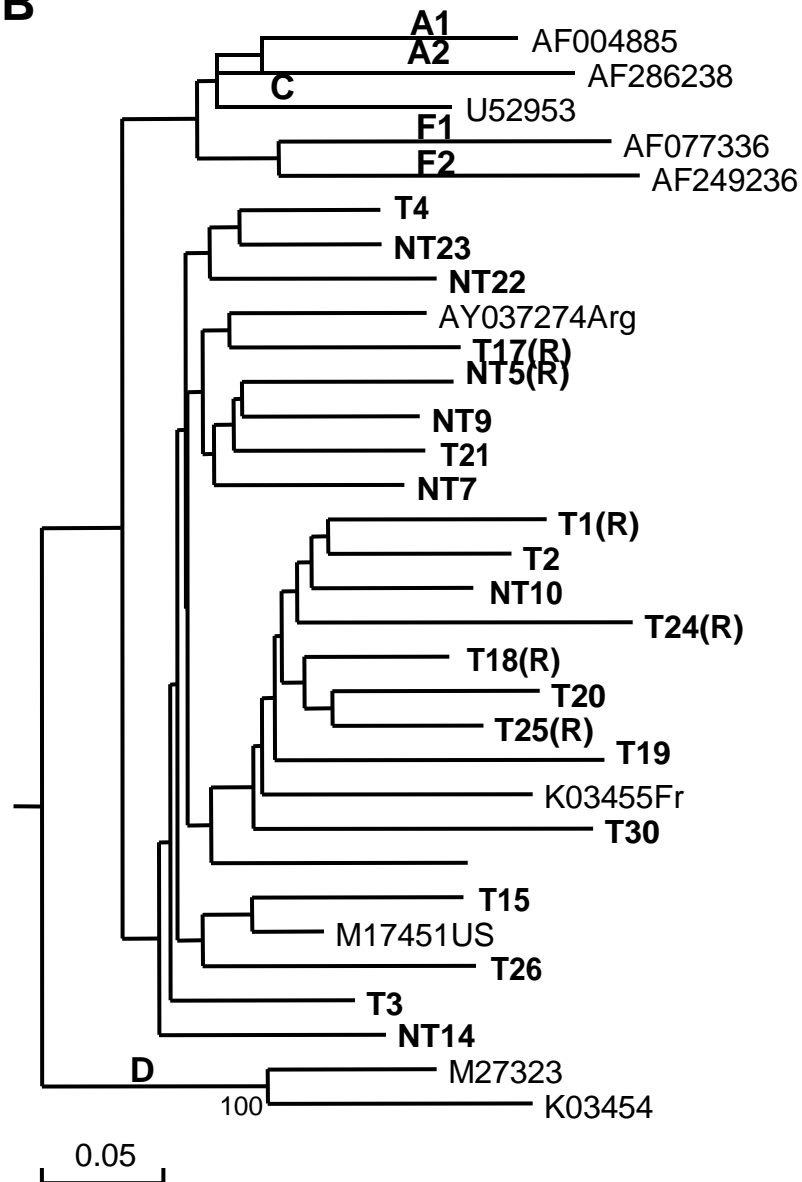
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## LEGENDS

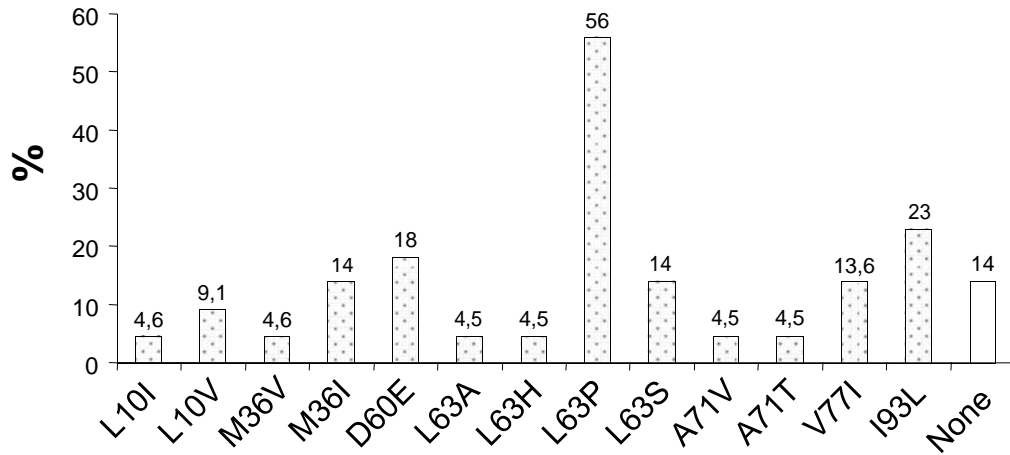
**Figure 1: Phylogenetic analysis of RT (A) and Env (B) regions.** Sequences obtained in this study are displayed in bold, with an R in the case of exhibiting resistance. Isolates are designated by their GenBank accession number and the name of the country of origin for subtype B sequences, except for Venezuelan isolates, which are shown in bold, with an R in the case of exhibiting resistance. Bootstrap values over 80% are shown in the tree. The scale represents the number of substitutions/site/100 bases. Letters in bold in the tree indicate subtype.

**Figure 2: Frequency of mutations associated to resistance to Pr, NRTI and NNRTI.**

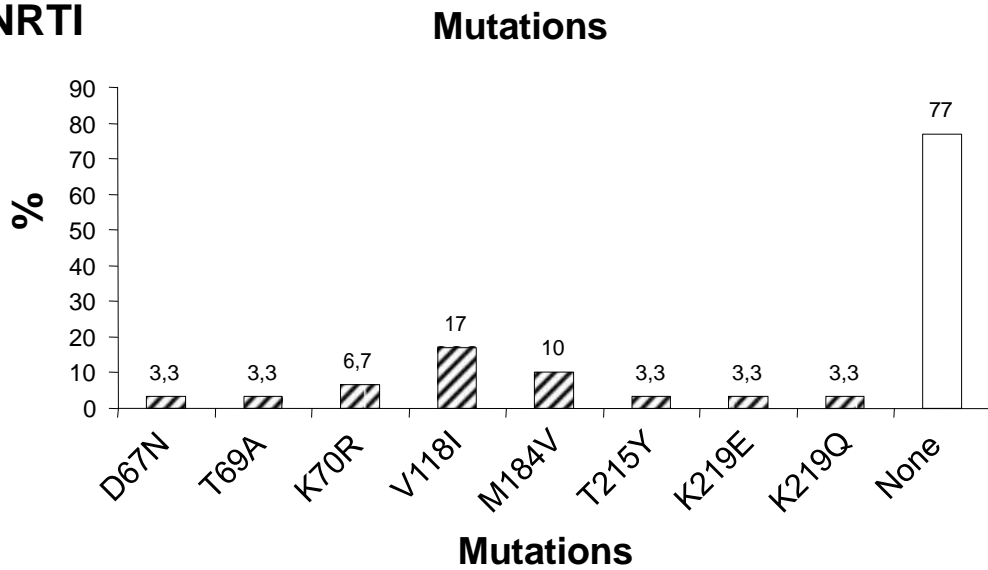
 : Primary mutations.  : Secondary mutations.  : Absence of mutations.  : K238T is a NNRTI-selected mutation that usually occurs in combination with other NNRTI-resistance mutations and which appears to confer intermediate levels of resistance to NVP and possibly DLV and efavirenz (EFV). In this HIV-1 isolate, the mutation was not found in combination with another NNRT resistance mutation.

**A****B**

### PrI



### NRTI



### NNRTI

