ANALYSIS OF HIV-TYPE 1 PROTEASE AND REVERSE TRANSCRIPTASE IN BRAZILIAN CHILDREN FAILING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)*

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SUMMARY

The aim of this study was to evaluate the genotypic resistance profiles of HIV-1 in children failing highly active antiretroviral therapy (HAART). Forty-one children (median age = 67 months) receiving HAART were submitted to genotypic testing when virological failure was detected. cDNA was extracted from PBMCs and amplified by nested PCR for the reverse transcriptase and protease regions of the pol gene. Drug resistance genotypes were determined from DNA sequencing. According to the genotypic analysis, 12/36 (33.3%) and 6/36 (16.6%) children showed resistance and possible resistance, respectively, to ZDV; 5/36 (14%) and 4/36 (11.1%), respectively, showed resistance and possible resistance to ddI; 4/36 (11.1%) showed resistance to 3TC and D4T; and 3/36 (8.3%) showed resistance to Abacavir. A high percentage (54%) of children exhibited mutations conferring resistance to NNRTI class drugs. Respective rates of resistance and possible resistance to PIs were: RTV (12.2%, 7.3%); APV (2.4%, 12.1%); SQV(0%, 12.1%); IDV (14.6%, 4.9%), NVP (22%, 4.9%), LPV/RTV (2.4%, 12.1%). Overall, 37/41 (90%) children exhibited virus with mutations related to drug resistance, while 9% exhibited resistance to all three antiretroviral drug classes.

KEYWORDS: HIV resistance; Antiretroviral therapy; Children; Treatment failure.

INTRODUCTION

The main goal of antiretroviral therapy is to suppress HIV replication to the greatest extent for as long as possible. Combination drug protocols for effective viral suppression require multiple daily doses of three or more medications over an indefinite period (HAART). Despite some progress in antiretroviral therapy, the lack of adequate pediatric formulations for certain drugs, together with maintaining the treatment regimen, have created a challenging situation for pediatricians providing treatment to infants and young children.

In Brazil, antiretroviral drugs are provided free of cost by the health system to HIV-1 infected patients. The use of the three-drug combination therapy with children was begun in 1997 at our clinical practice, and although we noted prolonged survival of the pediatric population6,10, we encountered other serious issues such as drug resistance. The emergence of HIV resistant strains during antiretroviral therapy is one of the main reasons for treatment failure in HIV-infected children3. Resistance of HIV-1 to antiretroviral agents results from mutations within the pol gene, which encodes for the viral reverse transcriptase (RT) and protease (Pt) regions, targets of currently used antiretroviral agents. In the present study, we analyze the genotypic resistance profiles of HIV in Brazilian children failing highly active antiretroviral therapy (HAART).

METHODS

The current study was conducted at Federal University of São Paulo, São Paulo, Brazil, between May 2000 and June 2001. The medical record review of 160 HIV-infected children attending the outpatient clinic at the Hospital Municipal de São Paulo was performed. Of these children, 41 were included in the study based on virological failure and written informed consent by the children’s caregivers. The Institutional Review Board of the Federal University of São Paulo approved the study, and informed consent was obtained from the caregivers of all included children.
Analysis of drug-resistance mutations: Consensus guidelines for resistance testing\(^{9}\) were used to define well-characterized, drug-resistance mutations. These mutations and the drugs to which they are related are (primary mutations are denoted by an asterisk): Ritonavir (RTV), K20M/R, V32I, L33F, M36I, M46I/L, I54L/V, A71T/V, V77I, V82A/P/S/T*\(^{,}\), I84V*, and L90M*; Nelfinavir (NVP) L10I, K30N*, M36I, M46I, G48V, A71T/V, V77I, I84V*, N88D, L90M*; Amprenavir (APV) V32I, M46I/L, I47V, I50*, I84V* Zidovudine (ZDV), M41L, D67N, K70R*, L210W, T215F/Y* and K219Q; Didanosine (ddI), K65R, L74V*, and M184I/V; Stavudine (d4T), V75T; 3TC, E44D*; and M184*; Nevirapine (NVP), L100I, K103N*, V106A*, V108I*, Y181C/I*, Y188C/H/L*, and G190A*; Efavirenz L100I, K103n*, Y188C/H/L*, P236L*; and multinucleoside, Q151M, 69 insertion. Strains with genetic mixtures of mutant and wild-type sequences at amino acid sites that code for major drug resistance were considered to be drug-resistant.

RESULTS

Thirty three of 41 children exhibited complete sequences from both the RT and Pt regions. Thirty-six sequences from the RT, and 38 sequences from the Pt regions were available for analysis. According to the genotypic analysis (GuideLines \(^{\text{TM}}\) Rules 5.0, Visible Genetics\(^{9}\), of 36 (33.3%) children showed resistance to ZDV, while 6 showed possible resistance to this drug (16.6%); 3/36 (14%) and 4/36 (11.1%) showed resistance or possible resistance, respectively, to ddI; 4/36 (11.1%) showed resistance to 3TC and D4T; and 3/36 (8.3%) showed resistance to Abacavir. A high percentage of children exhibited mutations conferring resistance to Nevirapine (44.4%) and Efavirenz (38.8%) (Fig. 1). Tables 2 and 3 show mutations related to decreased susceptibility to antiretroviral drugs and the antiretroviral therapy history, respectively. The major mutations related to PI resistance were D30N (n = 2); M46I (n = 3), V82A (n = 7) and L90M (n = 2), corresponding to the following respective rates of resistance and possible resistance to the different drugs: RTV (12.2%; 7.3%); APV (2.4%, 12.1%); SQV (0%, 12.1%); IDV (14.6%, 4.9%); NFV (22%, 4.9%); LPV/RTV (2.4%, 12.1%) (Fig. 2).

Quantification of HIV-1 RNA and T-cell subsets in peripheral blood: HIV-1 RNA copy numbers (viral load) were measured using a quantitative assay (NASBA, Biomérieux, France) with a lower quantitation limit of 80 copies/ml. T-lymphocyte subsets in peripheral blood were quantified by flow cytometry.

HIV-1 genotyping: DNA was extracted from PBMCs and amplified by nested PCR for the reverse transcriptase (RT) and protease (Pt) regions of the pol gene. Direct, bidirecional, dideoxynucleotide terminator cycle sequencing of the PCR product was performed using the OpenGene\(^{\text{TM}}\)DNA Sequencing System as described elsewhere\(^{3}\). Sequences were analyzed and manually proofread and edited.

Table 1

<table>
<thead>
<tr>
<th>Total of Patients (n)</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (months) (range)</td>
<td>66 (12-232)</td>
</tr>
<tr>
<td>Transmission route (n)</td>
<td>Vertical 38, Transfusional 3</td>
</tr>
<tr>
<td>Clinical category according to CDC*</td>
<td>A 12 (29.3), B 12 (29.3), C 15 (36.6), N 2 (4.9)</td>
</tr>
<tr>
<td>Laboratory Parameters at genotyping</td>
<td>Median, Range</td>
</tr>
<tr>
<td>Log(_{10}) viral load (copies/ml)</td>
<td>4.15, 2.1-6.4</td>
</tr>
<tr>
<td>CD4 (X 10(^{-6}) cell/L)</td>
<td>960, 52-2748</td>
</tr>
<tr>
<td>CD8 (X 10(^{-6}) cell/L)</td>
<td>1469, 411-7017</td>
</tr>
</tbody>
</table>

*CDC indicates Centers for Disease Control and Prevention

Fig. 1 - Incidence of resistance or possible resistance to NRTI and NNRTI in HIV-infected children failing HAART. ZDV= Zidovudine; 3TC= Lamivudine; D4T= Stavudine; ABC= Abacavir; NVP= Nevirapine; EFV= Efavirenz.
The percentages of patients with mutations related to drug resistance varied according to the drug classes employed: 57% for NRTI, 54% for NNRTIs, and 67% for PIs.

Three of 33 (9%) children with complete sequences from both the protease and reverse transcriptase regions of the pol gene showed resistance to all three drug classes (MDR), while 4/33 (12%) children showed no genotypic resistance at all.

By the time genotypic testing was performed, 19 children showed a PI-based HAART, while eight (42%) showed resistance to this drug class. Three of eleven children (27%) previously exposed to PIs and receiving NNRTI-based HAART were resistant to at least one PI.

**DISCUSSION**

Our analyses of HIV-1 from samples obtained at the time of virological failure revealed a high incidence of virus with mutations...
The major mutations associated with resistance to drugs not being used when the genotyping tests were performed included those related to Lamivudine (M184V; patient 10), or either Ritonavir or Indinavir (V82A; patients 5, 6, 20, 25, 33) and NNRTIs (K103N; patient 33). In some cases, these mutations may have been selected during a previous regimen and maintained by subsequent treatments\(^1\). However, this is unlikely to be the case for patients 10, 25 and 33, who exhibited principal mutations related to drugs to which they had not been previously exposed. In these three cases, a plausible explanation may be an uncommon cross-resistance phenomenon, such as M184V selected by DDI in patient 10, or V82A by Nelfinavir in patient 25, or less likely, primary resistance from mother-to-child transmitted resistant strains, which is probably the case for K103N in patient 33.

Only four children were NRTI naïve when HAART was begun and were experiencing their first therapeutic failures when submitted to genotypic testing (patients 13, 22, 30 and 35). Most children (90%) had been treated with antiretroviral nucleoside RT inhibitors before beginning of HAART. Consequently, many triple drug regimens included one or two NRTIs already used by the children, and this may be one of the reasons for difficulty in adequate suppression of viral replication and the subsequent failure of ARV treatment in this population.

Several findings in this study illustrate the consequences of sequential, non-potent regimens, with subsequent, sub-optimal responses to more potent schemes. Serial monotherapy with NRTIs may have selected for thymidine-associated mutations (TAM), along with resistance to ddi, ABC and 3TC. Conceivably by the time HAART was begun, truly potent, antiretroviral regimen for these children could not be developed due to cross-resistance to the “new” NRTIs. Not surprisingly, the responses to PIs or NNRTIs were transient, with selection of resistance mutations related to these drug classes. Our patients were extensively treated with Didanosine and Stavudine, although we found a low incidence of mutations at codons 74 and 75, consistent with other published reports\(^9\). Although the children in this study had never received ABC, a high percentage of resistance to this drug (8.3%) was found, according to one of the following rules from Guidelines rules 5.0\(^2\): NRTI 2, NRTI 3, NRTI 22. These findings again suggest cross-resistance.

Four children carried no resistance mutations in the context of a rising plasma HIV-1 RNA levels. In these cases, poor adherence to the treatment regimen should be thoroughly investigated, although cellular resistance, and/or the low, or theoretically, occasionally low, sensitivity of the test in detecting resistant strains (low negative predictive value) may be the cause\(^12\).

Our results confirm the close relationship between therapeutic failure and genotypic resistance in children, as also shown for adults\(^1,4,6,10\). The present study was not designed to assess whether the resistance found is the cause or the consequence of therapeutic failures. However, it is important to emphasize the possible interacting roles of adherence to the treatment regimen, regimen potency, and pharmacokinetics that may negatively influence the effectiveness of antiretroviral therapy in children.

Results like those shown here emphasize the importance of considering the appropriate moment at which to initiate ARV therapy, as well as the choice of adequate, suppressive, antiretroviral drugs. This may be especially important in children who exhibited a naturally high viral load compared to adults\(^2,11\). Thus, viral suppression with less potent regimens may not be effective considering the subsequent loss of efficacy in the succeeding regimens. The longer a child remains on a suboptimal suppressive regimen, the greater the likelihood of secondary mutations developing, and the greater the risk of subsequent, cross-resistance and drug failure\(^15\).
RESUMO

Análise da protease e transcriptase reversa do HIV-1 em crianças com falha terapêutica em uso de terapia anti-retroviral altamente eficaz (HAART)

O objetivo deste estudo foi avaliar o perfil de resistência genotípica do HIV-1 em crianças com falha terapêutica ao tratamento anti-retroviral (HAART). Quarenta e uma crianças (idade mediana = 67 meses) em uso de HAART foram submetidas ao teste de genotipagem no momento da detecção de falha ao tratamento. Foi realizada extração de cDNA de células periféricas mononucleares e amplificação do mesmo (regiões da transcriptase reversa e protease do gene de cDNA de células periféricas mononucleares e amplificação do no momento da detecção de falha ao tratamento. Foi realizada extração de cDNA de células periféricas mononucleares e amplificação do mesmo (regiões da transcriptase reversa e protease do gene pol) através de PCR-nested. O perfil genotípico foi determinado através do seqüenciamento de nucleotídeos. De acordo com a análise genotípica, 12/36 (33,3%) e 6/36 (16,6%) crianças apresentaram, respectivamente, resistência e possível resistência ao AZT; 5/36 (14%) e 4/36 (11,1%), respectivamente, eram resistentes e possivelmente resistentes ao ddI; 4/36 (11,1%) apresentaram resistência ao 3TC e D4T; e 3/36 (8,3%) eram resistentes ao ABC. Uma alta porcentagem de crianças (54%) apresentou mutações relacionadas à resistência aos inibidores da transcriptase reversa não-análogos de nucleosídeos. As taxas de resistência e possível resistência aos inibidores da protease foram, respectivamente: RTV (12,2%; 7,3%); APV (2,4%; 12,1%); SQV (0%; 12,1%); IDV (14,6%; 4,9%); NFV (22%; 4,9%); LPV/RTV (2,4%; 12,1%). No total, 37/41 (90%) crianças apresentaram vírus com mutações relacionadas à resistência a algum droga, sendo que 9% delas tinham vírus resistentes às três classes de drogas anti-retrovirais disponíveis.

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REFERENCES

7. GUIDELINES™ Rules 5.0 Available at: http://www.trugene.com/Physicians/ GuidelineRules_5.0.pdf.

