Lack of Evidence for Human T-Lymphotropic Virus type 1 and Mouse Mammary Tumor-like virus involvement in the genesis of Childhood Acute Lymphoblastic Leukemia


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Running title: HTLV1 and MMTV-LV in childhood leukemia.

Keywords: Childhood leukemia, HTLV1, MMTV, infectious etiology, molecular epidemiology.

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Abstract

Background: In Mexico City the incidence of childhood acute lymphoblastic leukemia (ALL) is one of the highest in the world, epidemiologic evidences suggest that infectious agents could be involved in the genesis of this disease. Early transmitted oncogenic retroviruses infecting lymphocytes are important candidates.

Methods: PCR based assays were used to screen viral genomic sequences of HTLV1 and MMTV-like virus (MMTV-LV) in leukemic cells from 67 pediatric patients with ALL.

Results: Viral genomic sequences were not detected in any sample by neither standard nor nested PCR.

Conclusions: Due to the methodological strictness and high statistical power of the study, these results suggest that HTLV1 and MMTV-LV are not involved in the genesis of childhood ALL in Mexican children.

Impact: To our knowledge this is the first work exploring the direct participation of HTLV1 and MMTV-LV retroviruses in childhood ALL development.
Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of childhood malignancy worldwide and Mexico City has one of the highest reported incidence rates at 49.5 cases per million (1). Identification of the causes that lead to the development of the disease remains a scientific challenge as discernible etiologic factors for almost all cases of primary leukemia are unknown. Several epidemiological studies have suggested that infections are involved, with different lines of evidence pointing to viral agents acting via direct oncogenic mechanisms; nevertheless, the identity of such agent(s) is presently unknown. This issue is reviewed elsewhere (2). Mexico City presents a higher incidence of infectious diseases than cities from developed countries and a recent study showed that serious infections in the first year of life were associated with increased risk of ALL in children with Down syndrome (3). The retroviridae family members are attractive candidates due to their promotion of oncogenesis through insertional mutagenesis and because they are known causative agents of leukemia in animals and humans. Human T-cell lymphotropic virus type 1 (HTLV1) is responsible for adult human T-cell leukemia (4), while, mouse mammary tumor virus (MMTV) is responsible for mouse breast tumors and lymphomas (5), and the participation of an MMTV like virus (MMTV-LV) has been suggested in human breast cancer. Both HTLV1 and MMTV infect B- and T- lymphocytes and can be transmitted intrauterine and by breast milk (4, 5). In this study, we assessed whether HTLV1 and MMTV-LV are involved in the genesis of childhood B-cell and T-cell ALL.

Materials and methods

The cases recruited in this study belong to the Mexican Inter-institutional Group for the Identification of the Causes of Childhood Leukaemia (MIGICCL), member of the Childhood
Leukemia International Consortium (CLIC) since 2012. 67 patients (61 of B-ALL and 6 of T-ALL) were enrolled during the period of the study (January 1 2010 to August 30 2012) Parents of enrolled patients signed a letter of consent and children older than 10 years also signed a letter of assent and were treated according to the ethical guidelines of our institution. All cases were from Mexico City, patient’s age ranged from 0.7 – 15.7 years (median = 7.6 years), gender distribution was 54% female. Diagnostic of precursor B- and T-cell ALL fulfilled the morphologic and phenotypic criteria. The average of leukemic blasts in bone marrow was 86% (range 25-100%). The screening of viral sequences was carried out using standard and nested PCR assays. DNA was obtained from bone marrow mononuclear cells fractions using QIAamp DNA extraction kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. All samples were subjected to β-actin gene amplification. PCR reactions contained 25-100 ng of DNA (first PCR) or 0.05 μl of product of first round PCR (nested PCR); PCR buffer contained 1.5-2.5 mM MgCl₂, 200 μM dNTPs, 200nM of each primer and 2.5 U of Taq Polymerase (Thermo Fisher Scientific, Waltham, MA). All primers used are described in table 1 and the amplification conditions are detailed in figure 1.

**Results**

We screened the presence of HTLV1 and MMTV-LV in bone marrow samples of childhood B- and T-cell ALL by PCR. The experimental approach to detect retroviral sequences was strict in several aspects (Fig 1a). Under these conditions we failed to detect positive samples for none of the retroviruses by standard and nested PCRs (Fig 1b and c). Since the study’s statistical power to detect ≥1 positive subject from our study population (N=67) from hypothetical 10% and 5% frequencies of infection had a confidence=99.99% and
95.00% respectively, then our results suggest that these retroviruses are not involved in the genesis of childhood ALL.

**Discussion**

To our knowledge, this is the first work exploring the direct participation of HTLV1 and MMTV-LV retroviruses in childhood ALL; however, other oncogenic viruses have been screened in leukemic cells, also with negative results (2). The investigation of the role of infectious agents in the etiology of childhood leukemia is based in distinct hypothesis arguing for both direct and indirect mechanisms; this work is based in the original Smith’s hypothesis for a direct causative role (2). The delayed infection hypothesis by Greaves argues for an indirect role for infections, however, epidemiological studies testing Greaves’ hypothesis have shown conflicting results (8). The different proposals about the infectious etiology of ALL have been tested by epidemiological, demographic and experimental studies founding the same variable results (8), population genetic traits could explain these differences. It is very likely that childhood leukemia is etiologically linked to infections; however, to demonstrate it, the use of new methodological strategies is the following and necessary step. For direct oncogenic mechanism, the list of candidate viruses is not exhausted; next generation sequencing is an attractive approach to ask for the presence of known and unknown infectious agents in leukemic cells.

**Acknowledgments**

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References


Table 1. Primers used to identify HTLV1 and MMTV-LV genomic sequences by standard and nested PCR

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<thead>
<tr>
<th>Virus (gene)</th>
<th>HTLV1 (tax) 5′→3′</th>
<th>MMTV-LV (env) 5′→3′</th>
<th>β-actin 5′→3′</th>
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<tbody>
<tr>
<td>First round 1</td>
<td>F CGGATACCCAGTCTACGTGT R CGATGGACGCGTTATCGGCTC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F CGAGCTAAGCGATTC R AGGTATGCCCACAGAT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F CCTAAGGCAACGCTGAAAAG R TCTTCATGGTGCTAGGAGCCA</td>
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<tr>
<td>First round 2</td>
<td>F TGTTCCCTAAACCGAATAG R GTGTGAGAGTAGAATGAGG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F CCTCACTGCCAGATC R CATCTGGCTGTGTAC&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Nested</td>
<td>F ACAAGCGAATAGAAGACTC R ATAAAGGAGGAGGACTGT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F GTATGAAAGCAGATGGGTAG R ATAGTCGTAGCAGAAGAG&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> These primers were designed for this study using the Basic Local Alignment Search Tool (BLAST).

<sup>b</sup> Since the presence of MMTV-LV in human breast cancer has been questioned, to reduce false positives, primers targeted regions with low identity to human endogenous retroviral sequences (7).

<sup>c</sup> Modified sequences of primers 2 and 4 reported in (7). F= forward and R= reverse.
Figure Legend

Figure 1. Experimental approach and viral genomic sequences screening in leukemic cells from pediatric ALL samples. a. To reduce the possibility of false negatives due to inadequate primer recognition (resulting from the high mutation rate occurring in retroviruses), we expanded the target region of amplification using two pairs of primers for each virus, additionally a nested PCR was implemented to confirm the first PCRs (top panel shows an schematic representation of primers target sites). Also, a gradient of annealing temperature was carried out to optimize every reaction, and the chosen annealing temperature was 55°C or less (white arrow) to increase the probability of primers hybridization even in the presence of mismatches (middle panel). The bottom panel shows a representative template gradient using DNA from positives controls. The bands correspond to the approximate signals we would observe in the samples considering that in ALL at least 25% of bone marrow cells are leukemic cells, then the limit of detection of the reactions was adjusted to equate at least 25% of infected cells (white arrow), fainter signals would indicate that the virus was not present in every leukemic cell.

b and c. Five (1-5) representative samples of first round PCR’s (top panels) to detect HTLV1 (b) and MMTV-LV (c) are shown; two different regions of HTLV1 tax and MMTV env genes were targeted (amplicons of 149 and 387 and of 284 and 666, respectively). For HTLV1 screening, DNA from MJ (ATCC, CRL-8293) and Daudy (D) (kindly provided by Dr. Mario Vega, Oncology Hospital at the National Medical Center XXI Century) cell lines were used as positive and negative controls, respectively. In the case of MMTV-LV screening we used DNA from BALB/c splenocytes (Bal) (kindly provided by Dr. K. Chávez, Pediatric Hospital at the National Medical Center XXI Century) as positive control. A reaction without DNA was run routinely (-). To discriminate molecular sizes, 100 bp molecular weight marker (M) (Thermo Fisher Scientific, Waltham, MA) was used. The
black arrows indicate the expected molecular size for every reaction. The amplification conditions were: 94°C (7'), followed by 35 cycles of 94°C (1'), 55°C (1') and 72°C (1') with a final extension step of 72°C (10'). Nested PCR for all samples was carried out and confirmed the previous negative results (b and c middle panels). The amplification conditions were: 94°C (5'), followed by 15 cycles of 94°C (45''), 55°C (45'') and 72°C (45'') with a final extension step of 72°C (10'). Amplification of β-actin endogenous gene was positive for all samples (bottom panels). The amplification conditions for β-actin were the same described for first round PCR’s for viral detection. The amplification products were subjected to electrophoresis in a 2% agarose gel stained with ethidium bromide and photographed under ultraviolet light using the Quantum ST4 System (VilberLourmat, Marne-la-Vallée, France).
Fig 1

(a) Schematic representation of the amplification products for (b) HTLV-1 and (c) MMTV-LV.

(b) HTLV-1

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(c) MMTV-LV

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