Cytosine arabinoside-metabolizing enzyme genes are underexpressed in children with *MLL* gene-rearranged acute lymphoblastic leukemia

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Abstract

Infant acute lymphoblastic leukemia (IALL) is characterized by mixed lineage leukemia (*MLL*) gene rearrangements, unique gene expression profiles, poor prognosis, and drug resistance. One exception is cytosine arabinoside (Ara-C) to which IALL cells seem to be more sensitive. We quantified mRNA expression of Ara-C key enzymes in leukemic lymphoblasts from 64 Brazilian ALL children, 15 of them presenting *MLL* gene rearrangement, and correlated it with clinical and biological features. The diagnosis was based on morphological criteria and immunophenotyping using monoclonal antibodies. *MLL* gene rearrangements were detected by conventional cytogenetic analysis, RT-PCR and/or fluorescence in situ hybridization. The *DCK* and *HENT1* expression levels were determined by real-time quantitative PCR using SYBR Green I. Relative quantification was made by the standard curve method. The results were analyzed by Mann-Whitney and Fisher exact tests. A P value of ≤0.05 was considered to be statistically significant. *DCK* and *HENT1* expression levels were significantly lower in children with *MLL* gene-rearranged ALL compared to children with *MLL* germ line ALL (P = 0.0003 and 0.03, respectively). Our results differ from previous ones concerning *HENT1* mRNA expression that observed a higher expression level in *MLL*-gene-rearranged leukemias. In conclusion, the expression of the genes related to Ara-C metabolism was lower in *MLL*-positive children in the sample studied, suggesting the presence of population differences in the expression profile of these genes especially for *HENT1*.

Introduction

There is evidence that specific leukemic subgroups may have distinct etiologies, and that molecular abnormalities associated with particular subgroups may be linked to specific causal mechanisms. Infant acute lymphoblastic leukemias (IALLs) display unique biological features (e.g., nearly 80% of infants with leukemia have a leukemic cell abnormality involving the mixed lineage leukemia gene, *MLL*) and clinical features (e.g.,
increased rates of treatment failure in infants compared to older children) that provide investigative models and important insights for the study of leukemogenesis (1).

Previously reported gene expression profiles have shown that ALLs possessing a rearranged *MLL* gene have a highly uniform and unique pattern that clearly distinguishes them from other ALL subtypes (2,3).

The optimum treatment for patients with *MLL* gene rearrangements remains to be identified. The very poor outcome of treatment for these patients, especially those diagnosed during infancy, has led to the recommendation of allogeneic hemopoietic stem-cell transplantation (4). A better understanding of the pharmacokinetics and chemosensitivity of IALLs may contribute to improving treatment and prognosis. Pieters et al. (5) showed in *in vitro* experiments that leukemic cells from infants with ALL are more resistant to prednisone and L-asparaginase and more sensitive to cytosine arabinoside (Ara-C) than cells from older children with ALL.

Ara-C is a nucleoside analogue which mimics physiological nucleoside uptake and metabolism resulting in cell death. Ara-C is taken up by cells mainly via the human equilibrative nucleoside transporter 1. Inside the cell, deoxyctydine kinase (dCK) primarily phosphorylates Ara-C into arabinoside monophosphate (Ara-CMP) and Ara-CMP is subsequently phosphorylated into its active cytotoxic form cytarabine triphosphate (Ara-CTP) until incorporation into DNA. For a standard-dose Ara-C, membrane transport capacity could be the rate-limiting step, but with high-dose Ara-C the activity of dCK may limit Ara-CTP formation (6).

To determine the clinical significance of mRNA expression of Ara-C-metabolizing enzymes in childhood ALL we quantified mRNA expression in leukemic blasts from 64 Brazilian children diagnosed with ALL by the real-time quantitative polymerase chain reaction (RQ-PCR). We also attempted to correlate its expression levels with some relevant clinical and biological features such as age, white blood cell (WBC) count at diagnosis, common ALL antigen (*CALLA*) expression, 11q23/*MLL* aberrations, and minimal residual disease level at day 28 of induction therapy.

**Material and Methods**

**Patients**

Written informed consent was obtained from the parents of all patients (including the ones with normal bone marrow, BM) at the time of enrollment, and all aspects of this investigation were approved by the Ethics Committees of the institutions involved in the study.

We analyzed BM samples from 44 consecutive untreated children with ALL from the University Hospital, Medical School of Ribeirão Preto, University of São Paulo. The samples were compared to those from 20 children aged less than 24 months (7 of them less than 12 months old) from the Brazilian Infant Leukemia Study Group coordinated by Instituto Nacional do Câncer, Rio de Janeiro, RJ, Brazil, between January 1998 and March 2004. Ten BM samples obtained from children without hematological disease, 5 children who underwent cardiac surgery for correction of congenital cardiopathies and 5 children who were BM donors, were used to determine normal BM expression. Only patients with normal hematological counts who exhibited no evidence of infections, genetic diseases, autoimmune disorders, renal, or hepatic dysfunctions were considered to be eligible. All samples contained at least 90% leukemic cells on light microscopy, and were morphologically classified as ALL according to French American British criteria (7) and immunophenotypically determined using a broad panel of monoclonal antibodies following EGIL classification (8). Samples were subjected to conventional cytogenetic analysis for the
presence of MLL gene aberrations, RT-PCR (standard and nested) for the presence of an MLL/AF4 fusion transcript [t(4;11) (q21; q23)] and tested by fluorescence in situ hybridization using a VYSIS LSI MLL probe (9). Gene fusion transcripts were detected with the standardized primer sets proposed by the BIOMED-1 Concerted Action (10). Detection of minimal residual disease (MRD) at day 28 of induction therapy was performed by PCR using consensus primers for the detection of a clonal population for TCRγ, IgH and TCRδ. This method can detect one leukemic cell in $10^{-2}$-$10^{-3}$ normal cells (11,12).

RNA extraction and cDNA synthesis

Total cellular RNA was extracted using the TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. An additional phenol-chloroform extraction was performed and isopropanol precipitation occurred at -20°C. The RNA quality was examined by gel electrophoresis. Following a denaturation step of 10 min at 70°C, RNA was reverse transcribed to single-stranded cDNA using a mix of random primers (3 µg/µL; Invitrogen). The reverse transcription reaction was performed in a total volume of 25 µL containing 0.2 mM of each dNTP (Invitrogen), 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen), and 25 U RNase-OUT (Invitrogen) at 37°C for 20 min, 70°C for 10 min, and 37°C for 60 min.

Quantitative real-time polymerase chain reactions

The mRNA expression levels of DCK, HENT1, and an endogenous housekeeping gene encoding for beta-glucuronidase (GUSB) as a reference were quantified using real-time PCR analysis (SYBR green I dye) on a Gene Amp® 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) (13). Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). All primers employed were cDNA specific and were purchased from Invitrogen (São Paulo, SP, Brazil). DCK and HENT1 designed primers were: a) DCK forward primer, TGCAGGGAAGTCAACATT; DCK reverse primer, TCCCACCATTCTTCTGAG; b) HENT1 forward primer, TGTTTCCAGCGTGA; HENT1 reverse primer, CAGGCCACATGAATACAG and GUSB primer were chosen after the evaluation performed by the Europe Against Cancer program (14); c) GUSB forward primer, GAAAATATGTTGTTGGAGAGCTCATT; GUSB reverse primer, CCGAGTGAGATCCCTTTTTTA. The RQ-PCR was performed in duplicate in a total reaction volume of 20 µL containing 10 µL SYBR Green PCR Master Mix, 15 µM forward and reverse primers, 3.5 µL dH2O, and 40 ng cDNA from each patient as a template. Samples were heated for 10 min at 95°C and amplified for 50 cycles of 15 s at 95°C and of 60 s at 60°C. Blank and positive controls (calibrators) were run in parallel to determine amplification efficiency within each experiment. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Quantification was performed using the standard curve method. A serial dilution in dH2O of cDNA derived from a cell line RNA pool (K562-Lucena) used as calibrator was amplified to construct standard curves for both target and control genes. The slopes of the standard curves ranged from -3.2 to -3.9. For each patient sample, the amount of target and control gene was determined from the appropriate standard curve. The target amount was subsequently divided by the control gene amount to obtain a normalized target value and calibrated by the standard RNA sample (15).

Statistical analysis

Statistical analysis was performed using
the SPSS 10 Software (SPSS Inc., Chicago, IL, USA). Correlation between \textit{DCK} and \textit{HENT1} expression was tested in association with some variables such as age (younger than 24 months versus older than 24 months), WBC count at diagnosis (less than 50 x 10^9/L versus more than 50 x 10^9/L), \textit{CALLA} surface antigen expression at diagnosis (positive versus negative), \textit{MLL} translocations and MRD at day 28 (positive versus negative) of induction therapy based on the non-parametric Mann-Whitney test and Fisher exact test. In the Fisher exact test analysis, these variables were evaluated by considering the median of the values detected for \textit{DCK} and \textit{HENT1} gene expressions. A P value of \leq 0.05 was considered to be statistically significant.

**Results**

The demographic and laboratory characteristics of the children included in this study are shown in Table 1. There were 37 boys and 27 girls aged 0-204 months (median 41 months), 56 had B-lineage ALL (42 \textit{CALLA} +), 7 had T-lineage ALL, and 1 had ALL that was not immunophenotyped. Abnormalities involving 11q23/\textit{MLL} were detected in 15 patients, 4/7 (57%) of them less than 12 months old, 8/13 (61%) between 12-24 months, and 3/44 (7%) more than 24 months old, and 11/15 presented the \textit{CALLA}-immunophenotype. All children were classified and treated according to the guidelines of the Brazilian Cooperative Group for the Treatment of Childhood Leukemia (GBTLI-93 and GBTLI-99): 39 were classified as being at high risk of relapse and 25 as being at standard risk. Ten children without hematological disease for whom BM aspirates were used to verify standard \textit{DCK} and \textit{HENT1} expression were 3 boys and 7 girls aged 0-144 months (median 20 months).

The levels of \textit{DCK} expression were lower Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Infants(^a) (N = 20)</th>
<th>Older children (N = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male:female ratio)</td>
<td>1.5:1 (15 months)</td>
<td>1.3:1 (65 months)</td>
</tr>
<tr>
<td>Age (median)</td>
<td>65 months</td>
<td>65 months</td>
</tr>
<tr>
<td>CALLA positive</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>WBC count ≥50 x 10^9/L</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>MLL rearranged</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Survival status</td>
<td>50%</td>
<td>50%</td>
</tr>
</tbody>
</table>

\(^a\)Children younger than 24 months of age; \textit{CALLA} = common acute lymphoblastic leukemia antigen; WBC = white blood cell count; MML = mixed lineage leukemia gene.

<table>
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<tr>
<th>Table 2. \textit{DCK} and \textit{HENT1} gene expression by 64 patients with acute lymphoblastic leukemia.</th>
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</thead>
<tbody>
<tr>
<td>Patient characteristics</td>
</tr>
<tr>
<td>Normal bone marrow</td>
</tr>
<tr>
<td>ALL bone marrow</td>
</tr>
<tr>
<td>\textit{MLL} rearranged</td>
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<tr>
<td>\textit{MLL} germ line</td>
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<tr>
<td>Age under 24 months</td>
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<tr>
<td>24 months and older</td>
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<td>WBC &lt;50,000/mm(^3)</td>
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<tr>
<td>WBC ≥50,000/mm(^3)</td>
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<tr>
<td>\textit{CALLA} positive</td>
</tr>
<tr>
<td>\textit{CALLA} negative</td>
</tr>
<tr>
<td>\textit{TEL/AML1} positive</td>
</tr>
<tr>
<td>\textit{TEL/AML1} negative</td>
</tr>
<tr>
<td>MRD d28 positive</td>
</tr>
<tr>
<td>MRD d28 negative</td>
</tr>
</tbody>
</table>

Data are reported as median values with range in parentheses. ALL = acute lymphoblastic leukemia; MML = mixed lineage leukemia gene; \textit{CALLA} = common ALL antigen; WBC = white blood cell count; MRD = minimal residual disease.
in ALL MLL-positive children in both analyses (median 0.02 vs 0.38; P = 0.0003 and 0.002 by the nonparametric Mann-Whitney test and by the Fisher exact test, respectively), and HENT1 levels were also lower in MLL-positive children (median 0.02 vs 0.20; P = 0.03) by the nonparametric Mann-Whitney test. Also, no significant correlation was found by the Fisher exact test (P = 0.24) compared to children with MLL germ line ALL (Figure 2).

The 20 children younger than 24 months with ALL had lower levels of DCK expression (P = 0.005) and borderline HENT1 levels (P = 0.06 and 0.09 by the nonparametric Mann-Whitney test and the Fisher exact test, respectively) compared to older children. The levels of DCK and HENT1 expression were not compared in children younger than 12 months due to the small number of cases.

Only by the Fisher exact test was it possible to determine a correlation between increased DCK expression and the presence of the CALLA (P = 0.03), whereas this association was not detected by the nonparametric Mann-Whitney test (P = 0.07) or by any of the analyses carried out on the HENT1 gene.

For the remaining variables analyzed (initial WBC count and MRD at day 28 of induction) no significant correlation was detected for either the DCK or HENT1 gene in any analysis.

**Discussion**

Characterized by MLL gene rearrangements, unique gene expression profiles, and a poor prognosis, infant ALL is usually considered to be a separate subgroup of childhood ALL (1,16,17). Prognostic features play a critical role in directing therapy for ALL, and as ALL treatment has become more successful, most efforts have focused on identifying patients at a higher risk of treatment failure that can be targeted with more aggressive or differentiated types of therapy. The identification of genes associated

Figure 1. DCK and HENT1 mRNA expression in normal and in acute lymphoblastic leukemia (ALL) bone marrow (BM). Deoxycytidine kinase (DCK; left graph) and human equilibrative nucleoside transporter 1 (HENT1; right graph) mRNA expression levels in bone marrow of children without hematological disease (first columns) and in ALL bone marrow (second columns; P < 0.001 for both the DCK and HENT1 genes, according to the Mann-Whitney test). The horizontal lines of the pluses indicate the median values (1.58 vs 0.23 for DCK and 1.78 vs 0.07 for HENT1) and the filled circles indicate values for individual patients.

Figure 2. DCK and HENT1 mRNA expression in children with acute lymphoblastic leukemia (ALL) with MLL gene-rearranged and MLL germ line. DCK (left graph) and HENT1 (right graph) mRNA expression in ALL children with MLL gene rearranged and MLL germ line (median 0.02 vs 0.38; P = 0.0003 for DCK and median 0.02 vs 0.20; P = 0.03 for HENT1, according to the Mann-Whitney test). The horizontal lines of the pluses indicate the median values and the filled circles indicate values for individual patients.
with the process of chemotherapeutical drug metabolism is suggested as an important tool to predict ALL survival (18).

Both genes showed higher expression in BM of children with no hematological disease compared to children with ALL with median values of gene expression of 1.58 vs 0.23 for DCK and 1.78 vs 0.07 for HENT1. Although a similar pattern of expression has been described for normal BM from different lineages and maturation subpopulations and for BM from children with ALL, some genes have been shown to be preferentially expressed by the leukemic cells, suggesting an ectopic activation of these genes and consequently a specific regulatory mechanism of the disease (19). Since in the present study we did not analyze normal BM aspirates from different lineages and maturation subpopulations, these results should be considered with caution.

Previously reported results (20) have revealed that infants with ALL express significantly less DCK and more HENT1 mRNA compared to older children. The same differences concerning DCK and HENT1 expression were observed by microarray technology between MLL gene rearranged and MLL germ line cases (all ages) by the same group. A proper infant subgroup comparison could not be performed due to the small number of children less than 12 months of age enrolled in the present study. The different pattern of HENT1 expression verified in MLL gene-rearranged cases in the present study may be explained by factors such as the ethnic diversity of Brazilian children, which, however, would need further investigation to be confirmed. As a limitation of a retrospective study, there were no suitable samples available for cytotoxicity assays and/or for measuring Ara-CTP accumulation in the blasts to correlate in vitro Ara-C sensitivities to HENT1 and DCK mRNA expression. In addition, there are some methodological differences between the two studies to be considered such as technology used (microarray versus SYBR Green RQ-PCR in the MLL gene-rearranged analysis) and the internal reference gene. In the present study, the GUSB gene was used as an internal reference gene instead of the GAPDH gene. Some publications agree with the finding that GAPDH expression varies considerably and consequently may be an unsuitable reference for RNA transcription analysis (14,21).

The present results on DCK expression agree with those reported by Stam et al. (20). Previous reports indicated that the blast cells of infants may be more sensitive to Ara-C (5). These data suggest that DCK is not a rate-limiting factor in Ara-C metabolism in infant ALL cells. Another possibility, which was also raised by these investigators, could be that DCK is post-transcriptionally regulated and that, despite the lower mRNA expression, the amount of protein or the activity of the enzyme in infant ALL cells is comparable to, or even higher than in cells from older children with ALL (20).

Mansson et al. (22) compared DCK expression in cell lines and in samples from leukemia patients measured by RQ-PCR and semi-quantitative RT-PCR. Enzyme activity was measured by a radioactive substrate-based technique and Western blot was used to measure the amount of dCK protein. They observed that DCK mRNA levels were in agreement with the activity of the enzyme. This finding suggests that mRNA expression is related to the expression of the protein and the activity of Ara-C-metabolizing enzymes.

The expression of the genes related to Ara-C metabolism was lower in MLL-positive children in the sample studied, indicating the pharmacokinetics and biological diversity of ALL, especially in MLL-positive children. Further improvement in the outcome of these patients will require new therapies targeted at the specific genetic lesions of the leukemic cells.

Acknowledgments

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