Modern Diagnostic Approach of Acute Lymphoblastic Leukemia in Children and Adolescents – Experience of a Single Pediatric Hematology-Oncology Center

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ABSTRACT

The authors analyzed a cohort of 33 children and adolescents (<18 years of age) consecutively admitted in the Pediatric Clinic of Fundeni Clinical Institute, diagnosed with ALL (acute lymphoblastic leukemia) and treated in the period of time 09.01.2008-12.31.2011. They discuss the role of modern techniques for diagnosis and risk stratification (immunophenotyping, cytogenetics, molecular biology) and present the results of their analysis, aiming to establish useful correlations for clinical management of cases. Introduction of new diagnostic methods allows a better therapeutic approach of cases, leading to superior event-free survival (EFS).

Key words: children and adolescents, acute lymphoblastic leukemia, diagnosis, stratification of risk

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy in children, representing 25% of all the neoplastic diseases in people younger than 15 years [1]. The peak of incidence is between ages 2 and 3 years [1]. In
adolescents (15 to 19 years), ALL comprises only 7% of all cancers [2]. In the United States, it is estimated that 2500 – 3500 children are diagnosed with ALL every year [1]. The incidence appears to be increasing. In Europe it was reported a 1.4% increase in incidence from 1970 to 1999 [3].

The pathogeny of ALL is complex, but it was shown that genetic factors play an important role. Many congenital / familial genetic disorders confer a high risk to ALL (Table 1), but they are quite rare. The majority of children with ALL have instead acquired genetic abnormalities detected in leukemia clones (Table 2).

ALL is a biologically heterogeneous disease [9] arising at any stage of lymphoid differentiation and characterized by an arrest in maturation and uncontrolled proliferation of lymphoid cells (blasts).

Clinical manifestations depends on extent of bone marrow infiltration with blast cells, as well on extramedullary infiltration of leukemic cells. Hematologic manifestations reflect the extension of bone marrow with blast cells, that cause anemia, neutropenia; the signs and symptoms arising from these manifestations are pallor, fatigue, petechiae, bruising, bleeding and fever, the last one due to infections. Extramedullary infiltration by leukemic cells causelymphadenopathy, hepatomegaly and splenomegaly, which are usually asymptomatic. Bone pain due to periosteal involvement is present in about 25% of children with ALL. Children with central nervous system (CNS) involvement may present symptoms like headache, vomiting, and lethargy. Testicular infiltration (in approximately 2% of males) and rare involvement of other organs than CNS, liver, spleen, and lymph nodes can also occur (eg, skin, eyes, pleural space, ovaries) [1, 10, 11].

The gold standard of diagnosis is bone marrow aspirate/biopsy, a mandatory investigation used to confirm diagnosis, to evaluate the number of blasts present, and to establish the leukemic phenotype. The morphology of blasts using the French-American-British (FAB) criteria is no longer in favor, because the subgroups (Fig. 1) do not correlate with the lineage or risk category [9]. Immuno-
Phenotyping of blast cells is now considered a superior method of diagnosis, because the expression of cell surface markers depends on their lineage (B or T) and stage of maturation. B-cell precursor ALL (CD 10+, CD 19+, CD 20+) represents 80-85% of childhood ALL [4]. T-cell ALL (15-18%) is diagnosed by positivity for CDs 2, 3, 5, 7 and 8 [4, 10, 14]. Early T-cell precursor phenotype (CD 8+, CD 5dim) is considered as having poor prognosis [5]. Mature B-cell leukemia (Burkitt; 2%-3%) is characterized by specific surface and cytoplasmic immunoglobulins and negativity for TdT (terminal deoxynucleotidyl transferase) marker [4]. Lumbar puncture (LP) is performed in ALL-patients to assess for CNS involvement. Cerebrospinal fluid (CSF) cytospin preparations are used to assess for the presence of lymphoblasts and to categorize the patient’s CNS status at leukemia diagnosis, as follows [9]:

- CNS1: absence of blasts and WBC in the CSF <5/μL;
- CNS2: presence of blasts and WBC count less than 5/μL;
- CNS3: presence of blasts and WBC count greater than 5/μL if the LP was nontraumatic.

Chemistry panels, liver and renal function studies, coagulation studies, other tests, procedures or imaging studies are based on the patient’s clinical and current laboratory findings [1], [4], [10]. Chest radiograph is done to assess for the presence of mediastinal mass, most common in older children and in those with T-cell ALL. The presence of a mediastinal mass may signal a possible medical emergency (the risk of imminent respiratory arrest caused by compression of the trachea or a risk of superior vena cava syndrome). Other investigations are ultrasonography of testis, magnetic resonance imaging (MRI) of the brain in the presence of CNS signs/symptoms, a basic echocardiogram for assessing the risk of anthracyclines toxicity, etc [15].

Modern ALL treatment protocols (ALL IC-BFM 2002, Interfant-06) [16, 17] imply risk-based therapy, in order to reduce toxicity in patients with low-risk ALL and to indicate aggressive therapy for those with a high-risk of relapse (high-risk ALL) [18]. The clinical and laboratory features used for risk stratification are summarized in Table 3.

**Table 3. Risk stratification [1] of new diagnosed pediatric ALL**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Favorable</th>
<th>Unfavorable</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NCI (19) Risk Category</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>1 – 10 years</td>
<td>&lt;1 y and &gt; 10 y</td>
</tr>
<tr>
<td>WBC at diagnosis</td>
<td>&lt;50 x 10⁹/mL</td>
<td>&gt;50 x 10⁹/μL</td>
</tr>
<tr>
<td><strong>Phenotype</strong></td>
<td>B – lineage ALL</td>
<td>T – lineage ALL</td>
</tr>
<tr>
<td><strong>CNS status</strong></td>
<td>CNS 1</td>
<td>CNS 2, CNS 3</td>
</tr>
<tr>
<td><strong>Testicular disease</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Genetics</strong></td>
<td>Hyperdiploidy</td>
<td>Hypodiploidy</td>
</tr>
<tr>
<td></td>
<td>Trisomy 4, Trisomy 10</td>
<td>Trisomy 21</td>
</tr>
<tr>
<td><strong>Induction failure</strong></td>
<td>t(12;21)/ETV6-RUNX1</td>
<td>1(9,22)/BCR-ABL, MLL rearrangements, iAMP21</td>
</tr>
<tr>
<td><strong>End of induction MRD</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NCI = National Cancer Institute (US); WBC = white blood cell; CNS = Central Nervous System; iAMP21 = intrachromosomal amplification of chromosome 21; MRD = minimal residual disease

**Original study**

**Background**

The aim of this study was to identify the cytogenetic and molecular abnormalities in pediatric ALL, in correlation with evolution, treatment results and prognosis. The study was a descriptive and observational one.

**Objectives**

- Evaluation of clinical and biological features at diagnosis;
- Analysis of the immunophenotyping, cytogenetics and molecular data at diagnosis and correlation with clinical and biological profile of cases;
- Inclusion in specific risk groups standard risk (SR), intermediate risk (IR), and high risk (HR);
- Treatment outcomes according to risk groups;
- Evaluation of complications, relapses and causes of death;
- A comparison of our results with international studies;

**MATERIALS AND METHODS**

Patients were eligible for inclusion if their age at inclusion was under 18 years and if they were diagnosed and treated in Pediatric Clinic - Fundeni Clinical Institute in the period of time 09.01.2008-12.31.2011.

Exclusion criteria were:

- Presence of physiologic statuses which contraindicate the treatment (pregnancy, breast feeding);
- Refused Protocol/Refused Consent to the Treatment - by family or adolescent patients after they have read the Informed Consent;
- Initiation of cytostatic therapy in another medical institution and following the treatment for longer...

**Table 3. Risk stratification [1] of new diagnosed pediatric ALL**
Modern Diagnostic Approach of Acute Lymphoblastic Leukemia in Children and Adolescents

- CBC differential
- BMP – aspirate, trephine biopsy for morphology, cytochemistry, immunophenotyping, cytogenetics and molecular biology
- ESR, CRP
- Liver screening tests: ALT, AST, Bilirubin, GGT
- Renal function screening tests: urine, serum creatinine, urea, uric acid
- Glucose, triglycerides, cholesterol
- Serum and urinary electrolytes
- Viral infection markers: EBV, HSV, CMV, HAV, HBV, HCV, HIV 1/2
- Coagulation (PT, AP, INR, aPTT, fibrinogen, FDP, d-dimers)
- Blood group
- Bacteriology (as clinical indicated): blood cultures, nasal and pharyngeal swabs, urine culture, opacultures, skin and other focal cultures
- Thorax Rx (PA, LAT)
- Abdominal ultrasound
- ECG and echocardiography
- Lumbar puncture (cytology)

Table 4. Laboratory and imagistic investigations at diagnosis

- CBC differential
- BMP – aspirate, trephine biopsy for morphology, cytochemistry, immunophenotyping, cytogenetics and molecular biology
- ESR, CRP
- Liver screening tests: ALT, AST, Bilirubin, GGT
- Renal function screening tests: urine, serum creatinine, urea, uric acid
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- ECG and echocardiography
- Lumbar puncture (cytology)
Table 5. Panel of monoclonal antibodies for immunophenotyping

- Marker of progenitor cells: CD 34
- Pan-hematopoietic marker: CD 45
- B-cell markers: CD 10, CD 19, CD 20, CD 24, CD 38, s IgM, s IgLu and light chains
- T/NK cell marker: CD 1a, CD 2, CD 3, CD 4, CD 5, CD 7, CD 8, CD 16, CD 56, TCR γδβ
- Myelomonocytic markers: CD 11b, CD 13, CD 14, CD 15, CD 33, CD 64, CD 119
- Red cell line marker: Glycophorin A
- Platelets marker: CD 61

CD=Cluster of differentiation; TCR=T cell receptor

Table 6. ALL risk stratification according to IC-BFM 2002 Protocol

<table>
<thead>
<tr>
<th>Standard Risk Group (SR)</th>
<th>Intermediate Risk Group (IR)</th>
<th>High Risk Group (HR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 1-6 years and</td>
<td>Blasts number D8 &lt;1000/µL (PGR) and</td>
<td>Blasts number D8 &gt;1000/µL (PPR) or</td>
</tr>
<tr>
<td>Leucocyte count at diagnosis &lt;20 000/µL and</td>
<td>Age &gt;1 - &gt;6 years or</td>
<td>BM – M2, M3, D33 or</td>
</tr>
<tr>
<td>Blasts number D8 &lt;1000/µL (PGR) and</td>
<td>Leucocyte count at diagnosis &gt;20 000/µL and</td>
<td>Presence of t(4;11)/MLL-AF4 or</td>
</tr>
<tr>
<td>BM – M1, M2, D15 and</td>
<td>BM – M1, M2, D15 and</td>
<td>Presence of t(9;22)/BCR-ABL or</td>
</tr>
<tr>
<td>BM – M1, D33 (complete remission – CR)</td>
<td>BM – M1, D33 or</td>
<td>Patient in the IR group and BM – M3, D15 (induction failure)</td>
</tr>
<tr>
<td>SR criteria</td>
<td>but BM – M3, D15</td>
<td>and BM – M1, D33</td>
</tr>
</tbody>
</table>

PGR=Prednisone good responders; PPR=Prednisone poor responders; BM - M1/2/3=BM status according to morphology; D8,15,33=Day 8,15,33.

Table 7. Risk group definition according to Interfant 2006 Protocol

<table>
<thead>
<tr>
<th>Standard Risk (SR)</th>
<th>Medium Risk (MR)</th>
<th>High Risk (HR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MLL rearrangement</td>
<td>MLL status unknown or</td>
<td>MLL rearrangement and</td>
</tr>
<tr>
<td>MLL rearrangement and age &gt; 6 months (183 days)</td>
<td>Leukocyte count at diagnosis &lt;300 000/µL</td>
<td>Leukocyte count at diagnosis ≥ 300000/µL</td>
</tr>
<tr>
<td>MLL rearrangement and age &gt; 6 months (183 days) and</td>
<td>Leukocyte count at diagnosis &lt;300 000/µL</td>
<td>Leukocyte count at diagnosis ≥ 300000/µL</td>
</tr>
</tbody>
</table>

Treatment

We used for chemotherapy the ALL IC-BFM 2002 Protocol in patients aged 1-18 years, and Interfant-06 Protocol in infants [16, 17].

Statistical analysis

The data obtained from analysis of clinical sheets were submitted to methods of descriptive statistics. Using the IBM SPSS statistics 20, Epi Info and Microsoft Excel 2010, we computed relative frequencies and realized the graphic representations. In order to test statistical significance of differences we used the χ², log-rank tests and Cox proportional regression model [22].

RESULTS

Hereditary factors

We find in the families of our patients 4 cases having malignant proliferation in their histories: 3 grade 2 relatives (grandparents) with Hodgkin disease, non-Hodgkin malignant lymphoma and lung cancer respectively. Also, 1 grade 3 relative (grand-grandfather) had a non-specified malignancy.

Clinical picture

The main clinical signs and symptoms in our cohort of patients are shown in the Fig. 2.

Laboratory

CBC (complete blood count) at diagnosis revealed an association between regenerative anemia, leukocytosis, thrombocytopenia and presence of blasts in peripheral blood (Table 8).

We have to mention that only statistical correlation was the strong tendency to have significant bleeding at a platelet count less than 50 000/µL (RR=2,4561 and OR = 4,0749).

Blast morphology using FAB classification, was the
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following: ALL-L1 blasts in 27 patients (81.81%), ALL-L2 blasts in 5 patients (15.15%) and ALL-L3 blasts in one patient (3.03%) (Fig. 3).

The immunophenotyping of blasts cells is reveal in Table 9.

We want also to highlight that most of our patients demonstrate aberrant phenotypes (“leukemia” phenotype):

- In 7 from 20 patients with common B ALL (35%) we find co-expression of myeloid markers;
- All the 3 cases of pro-B ALL had aberrant expression of myeloid markers, one of them having also a translocation of MLL gene (his age being less than 1 year);
- One of the 2 cases of T cell-ALL had an aberrant patient’s age is presented in Table 10.

Distribution of immunophenotypes according to
phenotype (co-expression of B-cell markers and maturational asynchrony).

Cytogenetic abnormalities:
- The ploidy of blasts is depicted in Table 11, together with other numeric chromosomal anomalies;
- Molecular biology. In 21 patients (63.63%) we could not demonstrate specific chromosomal translocation; the rest of abnormalities are revealed in Table 12.

Correlation between clinical and biological characteristics and blasts immunophenotype and cytogenetics/molecular biology

We could establish some correlations between clinical and biological characteristics of our patients and the blast immunophenotype and genetic peculiarities:
- Patients with hyperleukocytosis at diagnosis (17; 51.51%) were included in ALL-T group (both cases in our study), as well in ALL-B group;
- B-common ALL were the most frequent, having tendency to have the onset in children aged 1-6 y and 6-9 y (14/20 patients with B-ALL, 70%) and have been associated with favorable prognostic factors (leukocyte count <20 000/μL at diagnosis, modest infiltrative syndrome, without CNS involvement, normal or hyperdiploid karyotype, and t(12;21)(ETV6-RUNX1) translocation);
- B-common ALL having t(9;22)(BCR-ABL) translocation – 3 patients in our cohort – had a belated onset (>10 yoa) and were associated with unfavorable risk factors, especially hyperleukocytosis;
- Pro-B and Pre-B ALL had the onset at younger ages (mostly <1 yoa), and have been associated with hyperleukocytose, massive organ infiltration, t(4;11)(MLL-AF4) translocation and hypodiploidy (2/3 cases) – all of these been factors for unfavorable prognosis. Unlike the data from literature, our patients did not have CNS involvement at diagnose.
- 2 patients with T-ALL had the onset in adolescence and mediastinal mass; both of them however had favorable prognostic factors, unlike the data from literature.

Allocation of patients to risk group is shown in Fig. 4. Correlations between blast immunophenotype and cytogenetics/molecular biology are summarized in Fig. 5.

**DISCUSSION**

Starting from the actual wide-spread belief that ALL in children is a very heterogeneous disease, the aim of this study was to validate the newer methods for diagnosis and risk stratification in a single Pediatric Hematology-Oncology Center.

The main deficiency of our study was the limited number of patients, which made difficult finding of valid statistical correlations.

However, we could make some observations presented in the following statements.

Clinical and hematological manifestation in our cohort of patients are similar to data from literature [10], [23], [24], but we have to emphasize the absence of initial CNS involvement in our patients, probably a random situation due to numerical limitations of our study.

Bone marrow (BM) puncture/aspiration and trephine biopsy remains the gold standard for positive diagnosis in pediatric ALL.

It is now more and more clear that FAB classification – based on blast morphology – is no longer able to distinguish inside the biological puzzle of children ALL the peculiarities which could drive the therapy and establish the most adequate prognosis.

<table>
<thead>
<tr>
<th>Table 11. Cytogenetic abnormalities</th>
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<tbody>
<tr>
<td><strong>Blast ploidy</strong></td>
</tr>
<tr>
<td>Hyperdiploid</td>
</tr>
<tr>
<td>46-50 chromosomes</td>
</tr>
<tr>
<td>&gt;50 chromosomes</td>
</tr>
<tr>
<td>Hypodiploid</td>
</tr>
<tr>
<td>40-45 chromosomes</td>
</tr>
<tr>
<td>&lt;40 chromosomes</td>
</tr>
<tr>
<td>Normodiploid</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other cytogenetic anomalies</th>
<th>Number of patients</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 Trisomy (Down)</td>
<td>1</td>
<td>3.03</td>
</tr>
<tr>
<td>Inversion of chromosome</td>
<td>6</td>
<td>18.18</td>
</tr>
<tr>
<td>• with normoploidy</td>
<td>4</td>
<td>12.12</td>
</tr>
<tr>
<td>• with hypodiploidy</td>
<td>1</td>
<td>3.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 12. Molecular biology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alterations</strong></td>
</tr>
<tr>
<td>Number of patients</td>
</tr>
<tr>
<td>Percentage (%)</td>
</tr>
</tbody>
</table>

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Also hyperleukocytosis at diagnosis is found in ALL-T group as well as B-ALL group and it was our impression that is not among the very reliable criteria for risk stratification.

Introduction of newer diagnostic methods (i.e. immunophenotyping, cytogenetics, molecular biology) allows better stratification of patients, much closer to biological characteristics of disease [23], [24].

The identification of aberrant (“leukemic”) phenotypes is important for minimal residual disease (MRD) assessment and – therefore – for better tailoring the treatment [14, 25-27].

The complex approach of ALL patients enabled us to identify some important correlations between biological features of disease and evolutive tendency, and – due to this ascertainment– to estimate prognosis of ALL [28-31]:

- **B common – ALLs**, most frequent in our cohort (66.6%) as well as in literature, have tendency to have onset in children aged 1-9 years (70%) and are associated with favorable prognostic factors (initial leukocyte count <20 000/μL, modest infiltrative syndrome, no initial CNS involvement, hyperdiploid or normal karyotype, and favourable translocations, like t(12;21)[ETV6-RUNX1]). However, B common – ALLs with t(9;22)BCR/ABL translocations, have a belated onset (> 10 year) and are associated with other unfavorable risk factors, especially initial hyperleukocytosis.

- **Pro-B and Pre-B ALLs** have the tendency to have onset at younger ages (mostly <1 year) and are associated with hyperleukocytosis, massive organ infiltration, hypodiploidy and unfavorable translocations involving MLL gene – all of these being factors of unfavorable prognosis. We have to mention that –in contrast to data from literature – our patients did not have CNS involvement at the onset.

- **T-cell ALLs** have the onset in adolescence, the most striking association findings being initial hyperleukocytosis and presence of mediastinal mass (± superior vena cava obstruction syndrome, or respiratory compromise) – all of these known as unfavorable factors. Nevertheless, we should mention that in our cases we did not find CNS involvement at the onset, and that our patients had also favorable prognostic factors, an observation hampered by small number of patients.

Cumulative data from our patients allowed us to stratify them according to ALL IC-BFM 2002 and Infant-06 Protocols Criteria which we use in our Clinic. Although the percentages of cases allocated to SR, IR and HR groups differs from those presented in the literature, the post-therapy outcomes are not far from those of other contemporary studies. We intend to present these results in a future paper.

We should also mention that we did not have access to newer diagnostic techniques (i.e. whole genome sequencing, microarray, proteomics, pharmacogenomics) and this situation is characteristic for many Centers in low and middle-income countries. The acquisition of these techniques will allow a better understanding of clonal heterogeneity of disease, a better stratification of patients and will permit a better correlation of therapy to biological specificity of ALL patients.

Validation of stratification and of treatment outcomes in correlation to individual risk will be possible only by effective participation in vast international randomized and statistically supervised Protocols. We have now the status of observer in ALL IC-BFM Protocols and we hope to access a higher rank in these studies.

**CONCLUSIONS**

In our study of 33 children and adolescents consecutively admitted and diagnosed as ALL in a single Pediatric Hematology-Oncology Center we were able to obtain similar results to other contemporary studies regarding the value of new methods (immunophenotyping, cytogenetics and molecular biology) for diagnosis and risk stratification of cases. These techniques allow us to better adapt therapy to the individual specificity of patients. Significant break-
throughs are expected only by introduction of newer diagnostic tools (i.e. newer generation sequencing, whole genome sequencing, microarrays, proteomics, pharmacogenetics), a difficult task nowadays in low and middle-income countries.

REFERENCES


