ABSTRACT

Background: Zeta-chain-associated protein (ZAP-70) is a 70kD adaptor protein that acts quickly after T cell activation to propagate signal. The role of ZAP-70 in T-cell function is well established, and in the previous years, this molecule was considered to be T-cell specific. More recent data have documented a role of ZAP-70 in B cells. Interest in ZAP-70 has grown since it has been shown, through gene expression profiling, that it is expressed in a subset of cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL).

Purpose: The aim of this study was to investigate the expression of ZAP-70 in leukemic blasts of 50 newly diagnosed patients of B-lineage acute lymphoblastic leukemia (ALL), and to assess the correlation between ZAP-70 expression and various prognostic factors and outcome.

Patients and Methods: This study included 50 pediatric patients with newly diagnosed B-lineage ALL. They were 28 males (56%) and 22 females (44%) presented to the Pediatric Oncology Department, National Cancer Institute, Cairo University, during the period from 2005 to 2007. The age range was 2 to 17 years with a mean of 8.58±5.8 years and median 8 years. All patients were subjected at presentation to a full clinical history and physical examination. Patients diagnosed with ALL were enrolled on St. Jude Total XV protocol: standard risk and low risk according to results of primary investigation. Immunophenotyping was done using monoclonal antibodies which were analyzed on Coulter XL (Panel included CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD22, Cytoplasmic µ, anti κ, anti λ, CD13, CD33, anti classII MHC and TdT). Cases were considered ZAP-70 positive when exhibiting a ZAP/GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) ratio ≥0.13.

Results: The study revealed expression of ZAP-70 in 5/50 cases (10%). There was no statistically significant relation between ZAP-70 expression and the following: age, Total Leukocytic Count, hepatomegaly and splenomegaly. There was a correlation however between ZAP-70-expression and sex. Four patients died of disease progression: one patient with positive ZAP-70 expression and 3 patients with negative ZAP-70 expression. Fifteen patients (30%) relapsed after achieving complete remission (CR) and 3 patients (6%) did not achieve CR. Four patients of those who relapsed had positive ZAP-70 expression. The 2.5 years DFS was 73.1% for negative ZAP-70 cases while it was 20% in positive ZAP-70 cases. There was a statistically significant difference between 2.5-year DFS and ZAP-70 expression (p=0.048). The Overall Survival at 2.5-years for negative ZAP-70 cases was 93.3% while it was 80% for positive ZAP-70 cases with p-value =0.27.

Conclusion: Our results show that in B-Lineage ALL, ZAP-70 expression correlates with a worse DFS and an increased relapse rate. Furthermore, these results raise the need of prospective trials to evaluate the possibility of designing new compounds targeting this protein.

Key Words: ZAP-70 expression – Acute lymphoblastic leukemia.

INTRODUCTION

Zeta-chain-associated protein (ZAP-70) is a 70kD adaptor protein that acts quickly after T cell activation to propagate signal. It is comprised of two SH2 domains that bind phosphorylated ITAMs (immune receptor tyrosine based activation motifs) on CD3 (cluster of differentiation 3) and are joined by an interdomain linking the two SH2 domains. This protein tyrosine kinase is present in both CD4+ and CD8+ T cells and ultimately promotes gene transcription and also has a role in apoptosis. Due to its importance in propagating the signal
from the TCR (T cell receptor): CD3 complex, ZAP-70 is essential for a proper and complete T cell response [1].

The role of ZAP-70 in T-cell function is well established [2], and in the previous years, this molecule was considered to be T-cell specific. More recent data have documented a role of ZAP-70 in B cell development [3]. Interest in ZAP-70 has grown since it has been shown, through gene expression profiling, that it is expressed in a subset of cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and identifies cases with unmutated immunoglobulin heavy chain (IGH) genes and relatively poor prognosis [4,5]. However, little is known about its expression in other lymphoma types or acute leukemias. Therefore the aim of this study was to investigate the expression of ZAP-70 in leukemic blasts of 50 newly diagnosed patients of B-lineage acute lymphoblastic leukemia (ALL), and to assess the correlation between ZAP-70 expression and various prognostic factors and outcome.

PATIENTS AND METHODS

This study included 50 pediatric patients with newly diagnosed B-lineage ALL. They were 28 males (56%) and 22 females (44%) presenting to the Pediatric Oncology Department, National Cancer Institute, Cairo University, during the period from 2005 to 2007. The age range was 2 to 17 years with a mean of 8.58 ± 5.8 years and a median of 8 years. All patients were subjected at presentation to a full clinical history and physical examination. Radiological and laboratory investigation included:

- CBC.
- Bone marrow aspirate at presentation, day 15 and day 28.
- Immunophenotyping was done using monoclonal antibodies which were analyzed on Coulter XL (Panel included CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD22, CD34, Cytoplasmic μ, anti κ, anti λ, CD13, CD33, anti classII MHC and TdT) [6].
- Chest X-ray.
- Abdominal ultrasound.
- CSF examination.
- Liver and kidney function tests.
- Reverse transcription-polymerase chain reaction (RT-PCR) was performed on all patients’ and controls’ RNA samples for detection of ZAP 70 expression, using RNA Extraction by QIAamp RNA Blood Mini Kit, Qiagen and GeneAmpGold RNA PCR Reagent Kit, Applied Biosystems, according to the manufacturer’s instructions. Patients diagnosed with ALL were enrolled on St. Jude Total XV protocol: standard risk and low risk according to results of primary investigation [7].

Peripheral blood/bone marrow EDTA samples were obtained for RNA preparation. Cases selected for the gene evaluation had ≤2% CD3-positive T cells contamination as analyzed by flow cytometry because ZAP-70 is normally expressed in T cells so, we should exclude T cells contamination. Cases were considered ZAP-70 positive when exhibiting a ZAP/GAP-DH (Glyceraldehyde-3-phosphate dehydrogenase) ratio ≥0.13 (Fig. 1) [8].

Statistical analysis:

Data management and analysis were performed using Statistical Analysis Systems Software. Numerical data were summarized using means and standard deviations. Categorical data were summarized as percentages. Comparisons between the groups with respect to numeric variables were performed using Mann-Witney test, a nonparametric test equivalent to the t-test. Comparisons between categorical data were done using the chisquare test or Fisher’s exact test for small sample size. The survival functions were estimated by the Kaplan and Meier [9] method and compared by the log rank test [10]. All p-values were two-sided. p-values <0.05 were considered significant. The overall survival (OS) was calculated from date of diagnosis or presentation until death or end of the study. Disease free survival (DFS) was calculated from date of achievement of complete remission until relapse or death.

RESULTS

Patients:

The study comprised 50 pediatric patients with newly B-lineage ALL. The age ranged from 2 to 17 years with a mean of 8.58 years. They were 28 males and 22 females with a male to female ratio of 1.27: 1. ZAP-70 expression was measured for all patients to assess its possible prognostic significance on treatment outcome.
The study revealed expression of ZAP-70 in 5/50 cases (10%). Liver was enlarged in 25/50 patients (50%): 21 patients with negative ZAP-70 and 4 patients with positive ZAP-70 expression \((p\text{-value}=0.349)\). Spleen was enlarged in 32/50 patients (64%): 28 patients with negative ZAP-70 expression and 4 patients with positive ZAP-70 expression \((p\text{-value}=0.642)\). There was no statistically significant difference between either hepatomegaly or splenomegaly and ZAP-70 expression.

**Outcome and relapse rate:**

From the 50 patients included in this study, four patients died of disease progression: one patient with positive ZAP-70 expression and 3 patients with negative ZAP-70 expression. Thirty-two patients did not encounter relapse (64%): 28 patients with negative ZAP-70 expression and 4 patients with positive ZAP-70 expression \((p\text{-value}=0.642)\). There was no statistically significant difference between hepatomegaly or splenomegaly and ZAP-70 expression.

**Disease free survival (DFS):**

The 2.5 years DFS was 73.1% for negative ZAP-70 cases while it was 20% in positive ZAP-70 cases. There was a statistically significant difference between 2.5-year-DFS and ZAP-70 expression \((p=0.048)\) (Fig. 2).

**Overall survival (OS):**

The OS at 2.5 years for negative ZAP-70 cases was 93.3% while in it was 80% for positive ZAP-70 cases with \(p\text{-value}=0.272\) and results were not significant (Fig. 3).

**Correlation of ZAP-70 expression with different prognostic factors:**

Table (1) shows the correlation between the ZAP-70 expression and the following prognostic factors:

1- **Sex:**

ZAP-70 was expressed in 5 patients. They were all females. This result was statistically significant with a \(p\text{-value}=0.012\).

2- **Age:**

For patients with positive ZAP-70 expression, the age ranged from 4 to 12 years with a mean of 7.4 and median 6 years. While the age range for patients with negative ZAP-70 expression was from 2 to 17 years, with a mean of 8.71 and median 8 years. The age was not statistically significant when compared with ZAP-70 expression \((p=0.66)\).

3- **Total leukocytic count (TLC):**

The mean value of TLC was 24.95 \((x10^3/cm^3)\) in negative ZAP-70 negative patients while it was 25.4 \((x10^3/cm^3)\) in positive ZAP-70 cases and the difference between both groups was not significant \((p\text{-value}=0.962)\).

4- **Immunophenotyping:**

All patients’ samples were B-Lineage ALL. Further immunophenotypic characterization was done for all samples except 5 samples. Results showed pro-B stage in 8.9% of cases, pre-B in 44.4%, CALLA positive in 44.4% and mature-B in 2.3% of cases. Regarding the samples that showed positive ZAP-70 expression: 2 cases (40%) were pre-B, 2 cases (40%) were CALLA positive and one case (20%) was mature-B.

The maturational antigen CD19 was expressed in 78% of cases and in 100% of samples with positive ZAP-70 expression \((p\text{-value}=0.573)\). CD22 was expressed in 72% of samples and in 100% of samples with positive ZAP-70 expression \((p=0.304)\). CD10 was in 60% of samples and in 60% of samples with positive ZAP-70 expression \((p=1)\). CD34 was in 30% of samples, and in 100% of samples with positive ZAP-70 expression \((p=1)\).
ZAP-70, a tyrosine kinase of the Syk/ZAP-70 family that plays a critical role in the signal transduction from the TCR, has been reported to be expressed in T and natural killer derived cells [1,11,12]. Recently, ZAP-70 expression has been shown to be expressed in mice pro/pre B cells, its presence being important for B-cell development [3]. Surprisingly, ZAP-70 expression has also been found in a mature B-cell-derived neoplasm as CLL [13], particularly in cases with unmutated IgVH genes [4,5,15], where it seems to contribute to enhancing the signal from the B-cell receptor (BCR) [16]. Recent reports indicated that ZAP-70 expression is not limited to CLL but can also be found in other hematologic malignancies, such as precursor B-cell ALL, mantle-cell lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma [17,18]. In this study, ZAP-70 was expressed in 5/50 (10%) of pediatric patients. This frequency is lower than other B-Lineage studies: Chiaretti et al., 2006 reported that among 95 of adult patients, 59% showed positive ZAP-70 expression in their peripheral blood [22]. Also, Crespo et al., 2006 found that among 29 samples (22 adults, 7 children), 56% expressed positive ZAP-70 expression [14]. And a recent study by Khorshied et al., 2008 on the Egyptian population, found that among 40 patients (16 adults, 24 children), 47.5% of patients expressed ZAP-70 [19]. The age in our study ranged from 2 to 17 years with a median of 8 years. The age of patients with positive ZAP-70 expression ranged from 4 to 12 years with a median of 6 years. We found no statistically significant difference between age and the ZAP-70 expression. This is in accordance with other studies that found no correlation between age and ZAP-70 expression in the pediatric population, however they reported a positive correlation between ZAP-70 expression and age among adult patients [19]. The lower frequency of ZAP-70 expression in this study may be attributed to the small number of patients involved. Also it can be attributed to the difference in procedure and sampling between our study and other studies. The expression of ZAP-70 in leukemic cells can be assessed by different methods, such as Western blotting, quantitative RT-PCR, immunohistochemistry, and flow cytometry [10,20]. In our study, reverse transcription-polymerase chain reaction (RT-PCR) was performed on all
patients’ RNA samples for detection of ZAP 70 expression, while, the European Research Initiative on CLL (ERIC) has launched an international project to standardize the flow cytometry technique to determine ZAP-70, including the use of directly labeled anti-ZAP-70 mono-clonal antibodies. Khorshied et al., 2008 in their study assessed ZAP-70 by flow cytometry. In this study, there was no correlation between TLC & ZAP 70 expression the TLC showed no statistically significant difference when compared to ZAP-70 expression, however Khorshied et al., 2008 found a statistical significant difference between TLC and ZAP-70 expression, as well as the bleeding tendency in the form of purpuric eruptions and easy bruising [19]. Also, there was no correlation between hepatomegaly, splenomegaly and ZAP-70 expression.

Regarding sex, there was a statistically significant difference between sex and ZAP-70 expression. All positive ZAP-70 patients were females. In a recent study on B-Cell CLL, there was a trend of association between female sex and low Rai stage however there was no significant correlation between sex and ZAP-70 positivity [21]. Contrary to our findings, Khorshied et al., 2008 found no statistical significant difference between Zap-70 expression and sex in children and adults [19].

Regarding outcome, all patients were enrolled on the same protocol, thus uniformly treated. The 2.5-years DFS was 73.1% for negative ZAP-70 patients, while it was only 20% for positive ZAP-70 patients. Four of the five patients with ZAP-70 expression relapsed after achieving CR. These results indicate that ZAP-70 expression correlates with a shorter relapse-free survival after achievement of CR. These findings are in accordance with Chiaretti et al., 2006 [22], and similar to the results obtained in CLL, where high levels of ZAP-70 expression have been detected in patients with an unmutated status of the Ig variable region genes (IgVH), who are usually characterized by a more aggressive clinical course and often require therapeutic intervention [23-27]. Regarding immunophenotyping, results showed that ZAP-70 was expressed in 2/18 (10%) of pre-B samples. This is contrary to the results found by Chiaretti et al., 2006 and Khorshied et al., 2008 who found that ZAP-70 was expressed in 56% of patients with pro/pre phenotype [14]. In this study, ZAP-70 was expressed in 1/1 (100%) of mature-B samples. Similar results were reported by other authors [17,18, 19,22], ZAP-70 was expressed in 2/18 (10%) of CALLA samples. This is also similar to the results by Khorshied et al., 2008 [19] while Chiaretti et al., 2006 [22] found ZAP-70 expression in 52% of CALLA patients. The difference in results between our study and others may be attributed to the small number of samples expressing ZAP-70.

Conclusion:
In conclusion, our results show that in B-Lineage ALL, ZAP-70 expression correlates with a worse DFS and an increased relapse rate. Furthermore, these results raise the need of prospective trials to evaluate the possibility of designing new compounds targeting this protein.

REFERENCES


