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Expression and Clinical Implication of ZAP-70 in Acute Lymphoblastic Leukemia

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The purpose of this study was to investigate the expression of ZAP-70 in leukemic blasts of 40 newly diagnosed patients of B-cell acute lymphoblastic leukemia (B-ALL) by flow cytometry and to correlate its expression with the clinical and laboratory data as well as the response to induction chemotherapy together with CD20 as a maturational antigen (marker). ZAP-70 was expressed in 47.5% of cases (50% in adults and 45.8% in children). The highest expression was associated with pre and mature subtypes of B-ALL. ZAP-70 was significantly higher among the CD20 and Igμ positive cases, also it was associated with poor response to therapy especially in children, so it may have a prognostic value. Moreover, ZAP-70 could be a candidate molecule for targeted therapies in ALL.

Key words: ZAP-70, CD20, Igμ, B-cell acute lymphoblastic leukemia, flow cytometry

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INTRODUCTION

The Zeta-associated Protein (ZAP) is a 70 kDa molecule associated with the zeta (ζ) chain of the CD3 receptor complex of the lymphocyte population and belongs to the syk (spleen tyrosine kinase) family of tyrosine kinases. ZAP-70 protein is encoded by ZAP70 gene on long arm of chromosome 2 (2q12) (Verhoeyen *et al*., 2003). It is a cytoplasmic protein which is critically required for successful T-lymphocyte activation (Chan *et al*., 1992).

ZAP-70 has been considered a tyrosine kinase exclusively associated to the thymocyte, T and Natural Killer (NK)-cell compartments (Chan *et al*., 1992). ZAP-70 appears to be essential for both differentiation and maturation of pre-T cells and activation of mature T lymphocytes. Recent studies confirmed that ZAP-70 was important not only for CD4 but, also for CD8 T-cell development (Palacios and Weiss, 2007). Humans lacking ZAP-70 were found to have no $CD8^+$ T cells in their thymus and their mature peripheral blood T cells were non-responsive to antigenic stimulation (Chan *et al*., 1994).

The role of the ZAP-70 molecule in B-cell development has been established in murine model (Schweighoffer *et al*., 2003). This showed that this protein is also expressed in B-lineage cells and that it appears to play a role in their early development in particular during the pro-B to pre-B transition stage (Crespo *et al*., 2006). Recently, ZAP-70 was detected in normal B-cells from the human tonsils but not from the peripheral blood (Cutrona *et al*., 2006). In addition, it was found to be expressed in the $CD34^+$ normal bone marrow compartment including earlier B-cell progenitors, but not in $CD34^-$ pre-B and immature B-cells (Guillaume *et al*., 2005).

Although ZAP-70 is expressed exclusively in T cells, thymocytes and natural killer cells, the surprising expression of this T-lineage gene in chronic lymphocytic leukemia (CLL) cells, which drive from the B-cell lineage, has been confirmed (Chen *et al*., 2007). Chiaretti *et al*. (2006) studies revealed high expression of ZAP-70 in T-ALL. Moreover, in B-ALL, the levels of ZAP-70 expression increase along with the maturation process of the leukemic cells.

The present study aimed at evaluation of the expression of ZAP-70 by flow cytometry in 40 *de novo* B-ALL patients and to correlate these results with the clinical and laboratory data of the patients together with the response to induction chemotherapy.

MATERIALS AND METHODS

This study included 40 B-linage, newly diagnosed ALL patients, the patients were admitted to the National Cancer Institute and to Nuclear Medicine Department, Faculty of Medicine, Cairo University. This study started in October, 2006 and ended in February, 2008. The patients were 20 males and 20 females, aged from 2-34 years with a mean value of 13.4±9.5. The diagnosis was based on full history taking, clinical examination, morphologic examination of peripheral blood and bone marrow films (smears) and cytochemical studies (mainly for myeloperoxidase). Immunophenotyping of peripheral blood and/or bone marrow samples for surface expression of CD10, CD19, CD20, CD22, CD34, CD3, CD7, CD13 and CD33 (Becton Dickinson, Beckman Coulter, Fullerton, CA) to confirm the diagnosis and for proper sub typing of B-ALL.

For intracytoplasmic μ chain immunohistochemical staining, peripheral blood or bone marrow smears on positively charged slides were air dried for 4 h then fixed in absolute methanol at -70°C for 1 min. The primary monoclonal antibody used was IgM (Mu-Heavy chain-Ab-3-mouse monoclonal Ab (Clone R1/69), Catalogue number MS 1894-R7) (Lab Vision) and then detection with ultra vision detection system kit (anti-polyvalent, HRP/DAP ready to use for histological staining, Lab Vision Corporation (Catalogue number TP-015-HD). The proportion of intracytoplasmic μ cells was evaluated by light microscopy with oil immersion (original magnification x 1000) examining 500 cells per sample. Cases were considered as intracytoplasmic μ positive (brownish staining of the cytoplasm) if at least 20% of the mononuclear cells were positive as described by Crespo *et al*. (2006).

Expression of ZAP-70 by flow cytometry: One to two milliliter of bone marrow was aspirated under aseptic conditions through sternal or anterior superior iliac spine punctures according to the age of the patient. The aspirated marrow was added to sterile trisodium EDTA vacutainers. Blood is then diluted with Phosphate Buffered Saline (PBS) and layered over Ficoll hypaque for density gradient separation of the mononuclear cells. Bone marrow mononuclear cells were examined for the cytoplasmic expression of ZAP-70 using monoclonal antihuman ZAP-70, FITC conjugated (DAKO, 11-6695-80) and Zap-70 expression of more than 20% of the gated population is considered as positive result according to Chiaretti *et al*. (2006).

Therapeutic regimen of ALL: All pediatric patients less than 18 years received BFM 90/95 protocol for treatment of ALL. This protocol depends on risk stratification of patients into standard, medium and high risk groups. The main criteria for stratification was the leukemic cell mass estimate, initial response to steroids, presence of T-cell immunophenotype, t (9; 22) and CNS involvement. The

leukemic cell mass estimate was calculated by the following equation:

BFM-RF = 0.2×log (blood blast μL^{-1}+1)+ 0.6×liver size in cms below the costal margin×spleen size in cms below the costal margin

- Standard Risk Group (SRG) had fewer than 1000 μL^{-1} blasts in peripheral blood on day 8, a leukemic mass below 0.8, no CNS disease and no T-ALL or mediastinal mass
- Medium Risk Group (MRG) had fewer than 1000 μL^{-1} blasts in peripheral blood on day 8, a leukemic mass of 0.8 or higher and CNS disease or T-ALL or mediastinal mass
- High Risk Group (HRG) had more than 1000 μL^{-1} blasts on day 8 or less than 1000 μL^{-1} blasts in peripheral blood but 5% or greater marrow blasts on day 33 or Philadelphia positive ALL

Treatment consisted of induction, consolidation and re-induction for SRG and MRG. However HRG was treated by induction and intensive reconsolidation. CNS prophylactic radiotherapy of 1200cGy was given in MRG and HRG only.

As for adult patients they were all treated by one ALL adult protocol. This consisted of induction, consolidation, maintenance one and two. Induction contained weekly vincristine 1.5 mg m^{-2}, doxorubicin 25 mg m^{-2} for 3 weeks along with prednisone 60 mg day^{-1}. L-asprginase 10000 IU was given three times weekly along with the intrathecal injection of methotrexate 15 mg given weekly.

Follow up of the leukemic patients was carried out to study any possible association between the response to induction chemotherapy and ZAP-70 expression. The response to treatment was classified as follows according to Marie and Legrand (1999).

- **Death during induction:** Death during or after the first course of therapy with aplastic or hypocellular marrow.

- **Complete Remission (CR):** Cellular marrow with blast cells ≤5%, peripheral blood picture shows a neutrophil count≥1.5×10^3 mL^{-1}, platelet count≥100×10^3 mL^{-1} and no evidence of leukemia in other sites.

- **Primary resistance:** Cellular marrow with >5% blasts or evidence of leukemia in other sites.

Statistical analysis: Data were summarized and presented in the form of mean, range and standard deviation as descriptive statistics. Descriptive statistics and statistical comparison were performed using the statistical software program SPSS (version 14). Group comparison was done using either a 2-sample test or analysis of variance (ANOVA test). Correlation analysis was evaluated using the Pearson coefficient. For all of the above mentioned statistical tests done, the threshold of significance is fixed at 5% level (p-value). Probability value (p-value) of more than 0.05 was considered non-significant, while p-value less than 0.05 indicated a significant result.

RESULTS

The present study included 40 newly diagnosed B-linage ALL patients. Twenty males and twenty females, their age ranged between (2-34 years) with a mean value of 13.4±9.5. Among the 40 B-ALL patients, 19 (47.5%) showed ZAP-70 expression while 21 (52.5%) did not (ZAP-70 negative). The expression of ZAP-70 by flow cytometry in positive cases ranged between (56-99%) with a mean value of 80%.

The number of patients with purpuric eruptions and easy bruising was significantly higher in ZAP-70 positive patients ($p = 0.048$). There was no statistical significant difference between them as regards the age, sex, clinical presentation (fever and weight loss), hepatomegaly, splenomegaly and lymphadenopathy (Table 1).

Regarding the laboratory data, the peripheral blood white cell count was significantly higher in ZAP-70 positive patients ($p = 0.031$) while the platelet count was significantly lower ($p<0.001$). There is a negative correlation between ZAP-70 expression and the degree of thrombocytopenia ($r = -0.613$). Significant heterogeneity of ZAP-70 expression was observed among the different immunological categories of B-ALL ($p = 0.001$). ZAP-70 was significantly higher in pre B-ALL (57.9%) and mature B-ALL (100%). ZAP-70 was significantly lower in Calla positive (common) B-ALL patients (10.5%).

Both CD20 and intracytoplasmic μ chain were significantly high in the ZAP-70 positive patients ($p<0.000$ and 0.039, respectively). The response to induction chemotherapy according to ZAP-70 expression differed significantly as patients who were ZAP-70 positive had a higher rate of adverse outcome (resistance to induction or death during induction) ($p = 0.002$) (Table 2).

Patients were subdivided according to their age into two groups according to Wiemels *et al.* (2001):

- **Group 1:** Childhood B-ALL
- **Group 2:** Adulthood B-ALL

Table 1: Comparison of ZAP-70 expression (positive and negative) in all patients regarding their clinical and laboratory data

Data type		ZAP-70 negative (n = 21)	ZAP-70 positive (n = 19)	p-value	Significance
Clinical data	Age (years)	12.1±7.75	14.87±11.23	0.53	NS
	Sex				
	Male	13/21 (61.9%)	10/19 (52.63%)	0.35	NS
	Female	8/21 (38.09%)	9/19 (47.36%)		
	Splenomegaly	8/21 (38.09%)	9/19 (47.36%)	0.75	NS
	Hepatomegaly	8/21 (38.09%)	4/19 (21.05%)	0.24	NS
	Lymphadenopathy	12/21 (57.14%)	11/19 (57.89%)	0.96	NS
	Fever	11/21 (52.38%)	9/19 (47.36%)	0.75	NS
	Bleeding	4/21 (19%)	10/19 (52.63%)	0.026	S
	Weight loss	10/21 (47.61%)	12/19 (63.15%)	0.32	NS
Laboratory data (Mean±SD)	Hb (g dL^{-1})	7.6±1.42	6.59±1.91	0.65	NS
	WBC (x10^3 cm^{-3})	15.7±15.58	29.75±7.27	0.051	S
	Platelet (x10^3 cm^{-3})	56.57±20.24	29.26±17.76	0.000	HS
	PB blast (%)	46.43±29	53.58±7.93	0.49	NS
	BM blasts (%)	89.62±19.49	87.42±24.6	0.75	NS
FAB subtype	L1	7/21 (33.33%)	7/19 (36.84%)	0.37	NS
	L2	14/21 (66.66%)	8/19 (42.10%)		
	L3	0/21	4/19 (21.05%)		
Immuno-phenotyping	Pro	5/21 (23.80%)	2/19 (10.52%)	0.003	HS
	Calla	10/21 (47.61%)	2/19 (10.52%)		
	Pre	6/21 (28.57%)	11/19 57.89%)		
	Mature	0/21	4/19 (21.05%)		
Intracytoplasmic μ chain	+	6/21 (28.57%)	15/19 (78.94%)	0.001	HS
	-	15/21 (71.42%)	4/19 (21.06%)		
CD 20	+	3/21 (14.28%)	9/19 (47.36%)	0.023	S
	-	18/21(85.71%)	10/19 (52.63%)		

+ = Present, - = Absent, S = Statistically significant, HS = Highly significant, NS = Statistically not significant

Table 2: Expression of ZAP-70 in relation to the response of induction chemotherapy in all patients

Response of all patients to induction chemotherapy	ZAP-70 negative (n = 21)	ZAP-70 positive (n = 19)	p-value	Significance
Complete Remission (CR)	18/21 (85.71%)	7/19 (36.84%)	0.002	HS
Resistant to induction	2/21 (9.52%)	6/19 (31.57%)		
Death during induction	1/21 (4.76%)	6/19 (31.57%)		

HS = Highly significant

Group 1 included 24 *de novo* B-ALL patients; 12 males and 12 females, their age ranged between (2-15 years) with a mean value of 6.7±4.1. ZAP-70 was expressed in 11/24 (45.83%) of cases. The peripheral blood white cell count was significantly higher in ZAP-70 positive patients (p = 0.050) while the hemoglobin level and platelet count were significantly lower (p = 0.029 and 0.016). There is a negative correlation between ZAP-70 expression and the degree of anemia and thrombocytopenia (r = -0.484 and -0.446, respectively) (Table 3).

Statistically significant difference was observed among the different immunological categories of B-ALL as ZAP-70 expression was significantly higher in mature ALL patients and significantly lower in calla positive patients (p = 0.019). Detection of intracytoplasmic μ (Fig. 1, 2) chain was significantly higher in the ZAP-70 positive patients (p<0.001). The response to induction chemotherapy stratified according to ZAP-70 expression differed significantly as patients who were ZAP-70 positive had a higher rate of adverse outcome (resistance to induction or death during induction) (p = 0.001) (Table 4).

Group 2 included 16 *de novo* B-ALL patients; 8 males and 8 females, their age ranged between (18-34 years) with a mean value of 23.4±5.2. ZAP-70 was expressed in 8/16 (50%) of cases. Comparison between ZAP-70 positive and negative patients is shown in Table 5. There is a statistical significant difference between the two groups as regards the age (p = 0.025). There is a positive correlation between ZAP-70 expression and age (r = 0.558). Lymphadenopathy and purpuric eruptions are significantly higher among ZAP-70 positive patients (p = 0.021 and 0.021).

The peripheral blood white cell count was significantly higher (p = 0.042) while the platelet count was significantly lower in ZAP-70 positive patients (p<0.001). There is a negative correlation between ZAP-70 expression and the degree of thrombocytopenia (r = -0.847). Expression of intracytoplasmic μ chain was significantly higher in ZAP-70 positive patients (p = 0.049). ZAP-70 positive patients showed higher rate of adverse outcome (resistance to induction or death during induction) yet it is statistically insignificant (p = 0.085) (Table 6).

Table 3: Comparison of ZAP-70 expression (positive and negative) in children patients regarding their clinical and laboratory data

Data type		ZAP-70 negative (n = 13)	ZAP-70 positive (n = 11)	p-value	Significance
Clinical data	Age (years)	6.85±4.48	6.6±4.75	0.88	NS
	Sex				
	Male	8/13 (61.5%)	4//11(36.36%)	0.28	NS
	Female	5/13 (38.48%)	7/11(63.63%)		
	Splenomegaly	5/13 (38.48%)	6/11 54.54%)	0.23	NS
	Hepatomegaly	2/13 (15.38%)	4/11 36.36%)	1.00	NS
	Lymphadenopathy	6/13 (46.15%)	5/11 45.45%)	0.97	NS
	Fever	5/13 (38.48%)	7/11(63.63%)	0.21	NS
	Bleeding	4/13(30.76%)	6/11(54.54%)	0.23	NS
	Weight loss	6/13 (46.15%)	8/11(72.72%)	0.18	NS
Laboratory data	Hb (g dL^{-1})	7.6±1.57	6.24±1.07	0.029	S
	WBC (x10^3 cm^{-3})	20.55±18.09	40.74±31.44	0.050	S
	Platelet (x10^3 cm^{-3})	51.85±25.03	28.5±16.72	0.016	S
	PB blast (%)	46.62±24.19	64.91±3.84	0.138	NS
	BM blasts (%)	97.46±262	98±1.8	0.38	NS
FAB subtype	L1	7/13 (36.84%)	4/11(36.36%)	0.12	NS
	L2	6/13 (46.15%)	4/11(36.36%)		
	L3	0/13	3/11(27.27%)		
Immuno-phenotyping	Pro	2/13(15.38%)	1/11 (9.09%)	0.019	S
	CALLA	7/13	1/11 (9.09%)		
	Pre	4/13 (30.76%)	6/11(54.54%)		
	Mature	0/13	3/11(27.27%)		
Intracytoplas-mic µ chain	+	4/13 (30.76%)	9/11(81.81%)	0.000	HS
	-	9/13(69.23%)	2/11(18.18%)		
CD 20	+	2/13(15.38%)	5/11(45.45%)	0.106	NS
	-	11/13 (84.6%)	6/11(54.54%)		

+ = Present, - = Absent, S = Statistically significant, HS = Highly significant, NS = Statistically not significant

Table 4: Expression of ZAP-70 in relation to the response of induction chemotherapy in children

Response of children patients to induction chemotherapy	ZAP-70 negative (n = 13)	ZAP-70 positive (n = 11)	p-value	Significance
Complete remission (CR)	11/13 (84.61%)	3/11 (27.27%)	0.011	S
Resistant to induction	1/13 (7.69%)	4/11 (36.36%)		
Death during induction	1/13 (7.69%)	4/11 (36.36%)		

S = Statistically significant

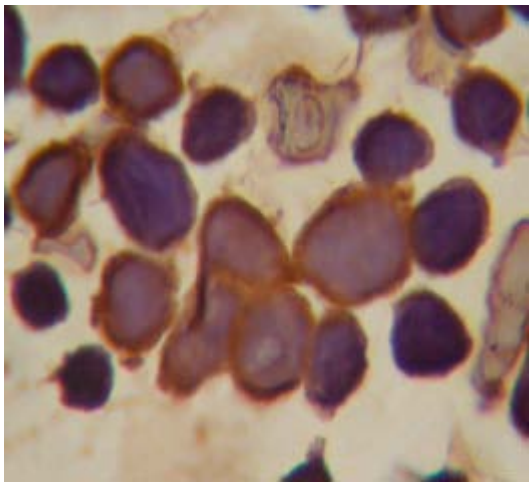

Fig. 1: ALL case showing intracytoplasmic µ chain positivity (brownish staining of the cytoplasm) by immunocytochemical staining

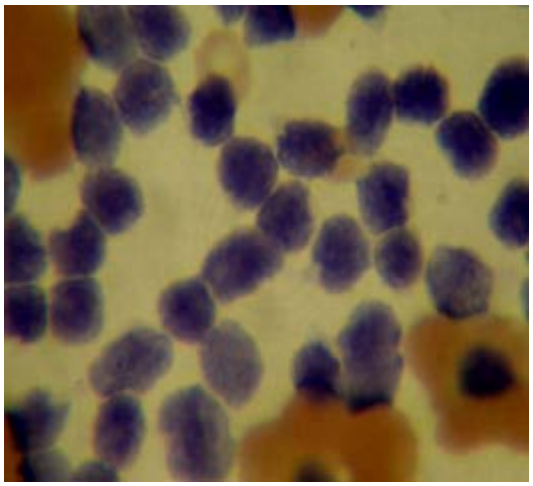

Fig. 2: ALL case negative for intracytoplasmic µ chain staining by immunocytochemical staining

Table 5: Comparison of ZAP-70 expression (positive and negative) in adult patients regarding their clinical and laboratory data

Data type		ZAP-70 negative (n = 8)	ZAP-70 positive (n = 8)	p-value	Significance
Clinical data	Age (years)	20.63±1.7	26.25±6	0.025	S
	Sex				
	Male	5/8 (62.5%)	6/8(75%)	0.59	NS
	Female	3/8(37.5)	2/8(25%)		
	Splenomegaly	2/8 (25%)	2/8 (25%)	1.00	NS
	Hepatomegaly	3/8 (37.5%)	0/8 (0 %)	0.055	NS
	Lymphadenopathy	0/8 (0 %)	4/8 (50 %)	0.021	S
	Fever	2/8 (50%)	2/8 (50%)	1.00	NS
	Bleeding	0/8 (0%)	4/8 (50%)	0.021	S
	Weight loss	1/8 (12.5%)	1/8 (12.5%)	1.00	NS
Laboratory data (Mean±SD)	Hb (g dL^{-1})	7.6±1.24	7.08±2.7	0.62	NS
	TLC (x10^3 cm^{-3})	7.83±4.27	14.65±7	0.042	S
	Platelet (x10^3 cm^{-3})	64.25±2.81	28.5±16.72	0.001	HS
	PB blast (%)	46.13±37.42	38±39.82	0.66	NS
	BM blasts (%)	76.88±27.31	71.5±32.25	0.71	NS
FAB subtype	L1	0/8 (0%)	3/8 (37.5%)	0.31	NS
	L2	8/8 (100%)	4/8 (50%)		
	L3	0/8 (0%)	1/8 (12.2%)		
Immuno-phenotyping	Pro	3/8 (37.5%)	1/8 (12.2%)	0.064	NS
	CALLA	3/8 (37.5%)	1/8 (12.2%)		
	Pre	2/8 (25%)	5/8 (62.5%)		
	Mature	0/8 (0%)	1/8 (12.2%)		
Intracyto-plasmic μ chain	+	2/8 (25%)	6/8 (75%)	0.046	S
	-	6/8 (75%)	2/8 (25%)		
CD 20	+	1/8 (12.2%)	4/8 (50%)	0.106	NS
	-	7/8 (87.5)	4/8 (50%)		

+ = Present, - = Absent, S = Statistically significant, HS = Highly significant, NS = Statistically not significant

Table 6: Expression of ZAP-70 in relation to the response of induction chemotherapy in adults

Response of adult patients to induction chemotherapy	ZAP-70 negative (n = 8)	ZAP-70 positive (n = 8)	p-value	Significance
Complete remission(CR)	7/8 (87.5%)	4/8 (50%)	0.086	NS
Resistant to induction	1/8 (12.5%)	2/8 (25%)		
Death during induction	0/8 (0%)	2/8 (25%)		

NS = Statistically not significant

DISCUSSION

We investigated the expression of ZAP-70 in the bone marrow and/or peripheral blood samples of 40 newly diagnosed B-cell ALL by flow cytometry and to correlate its expression with the clinical presentation, laboratory investigations specially CD20 and Igμ as maturation markers as well as the response to induction chemotherapy.

Crespo *et al.* (2003) have reported high expression of ZAP-70 in the peripheral blood mononuclear cells of all control subjects using flow cytometry and immunoblotting techniques. This can be explained by the normal expression of ZAP-70 by T-lymphocytes and natural killer cell populations which together represents 80-90% of peripheral blood lymphocytes. They also reported that ZAP-70 is not expressed in normal mature B-cells of the bone marrow. For such reasons, we did not have a control group in present study and according to Chiaretti *et al.* (2006) Zap-70 expression of more than 20% of the gated population by flow cytometry is considered as positive result.

In the present study, 19/40 B-cell ALLs (47.5%) were ZAP-70 positive and 21/40 (52.5%) were negative. This is in accordance with the results reported by Chiaretti *et al.* (2006) were (59%) of the patients included in their study showed positive expression of ZAP-70 in their peripheral blood. Also Crespo *et al.* (2006) reported that ZAP-70 was expressed by 56% of B-ALLs. These results indicate that ZAP-70 expression is not limited to chronic lymphocytic leukemia (CLL) but can also be found in other hematological malignancies, such as precursor B-cell-ALL. The degree of ZAP-70 positivity in this study may be slightly lower than that of Crespo *et al.* (2006) and Chiaretti *et al.* (2006), because of the higher number of children versus adult patients (children:adult ratio = 3: 2), as ZAP-70 was expressed in 50% of adult patients and in 45.8% of children while Chiearetti *et al.* (2006) study included 25 adults and 14 children (children:adult ratio = 1:2).

The flow cytometry analysis of ZAP-70 expression in B-ALL blast cells ranged between (25-96%) with a mean value of 75.16+21.79% in ZAP-70 positive cases, while the expression was considered low (1-18%) with a mean value

of 7.57+5.16% in negative cases. These results are in agreement with that of Crespo *et al.* (2006) were the level of ZAP-70 expression ranged between (56-99%) with a mean value of 80% in positive cases and 1-17% with a mean value of 7% in negative cases.

Comparing the clinical and hematological data of ZAP-70 positive and negative patients, a statistically significant difference was found between the two groups regarding the total leucocytic count as well as the bleeding tendency in the form of purpuric eruptions and easy bruising. This can be explained by the negative correlation between ZAP-70 expression and the degree of thrombocytopenia.

In group 1 (children), there is a statistically significant difference between ZAP-70 positive and negative patients as regard the degree of anemia and thrombocytopenia, both hemoglobin level and platelet count being lower in ZAP-70 positive patients. In group 2 (adults), comparing the clinical and hematological data of ZAP-70 positive and negative adult patients revealed that there is a positive correlation between ZAP-70 expression and age among adult patients. The incidence of generalized lymphadenopathy is significantly higher among ZAP-70 positive adults together with higher total leucocytic count. These findings could be explained by the higher percentage of blast cells carrying positive expression of ZAP-70 protein infiltrating the lymph nods, bone marrow and subsequently detected in peripheral blood and causing more tumor burden.

The expression of the maturational antigen CD20 and Igμ was significantly higher among the ZAP-70 positive patients than those with negative ZAP-70 expression. This is in accordance with the study of Chiaretti *et al.* (2006) in which ZAP-70 was consistently found in Igμ positive cases and there was higher expression of ZAP-70 in CD 20 positive patients than in patients with negative CD20 expression but these differences didn't reach statistical significance. These data clearly indicate that ZAP-70 expression increase along with the maturation process of the B-cell population. However, Crespo *et al.* (2006) reported that no relationship was observed between ZAP-70 expression levels and the maturation status of B-ALLs.

ZAP-70 was significantly expressed in pre-B ALL cases 11/19 (57.89%). Contrary to this, Chiaretti *et al.* (2006) reported that ZAP-70 was expressed in 5/6 (85%) of pre-B ALL patients. This may be attributed to the difference in patient's number in the two studies. Furthermore, Crespo *et al.* (2006) stated that ZAP-70 was expressed in 13 /23 (56%) of B-ALL patients with pro/pre phenotype. These results strengthen the data previously reported in mice, indicating that ZAP-70 plays a role in the process of pro-B to pre-B transition; its absence, combined with the absence of SYK, arrests the cells at the pro-B stage and induces a blockage of several pre-B-cell receptor (BCR)-induced events, such as differentiation into pre-B-cells, cell proliferation and heavy chain allelic exclusion (Schweighoffer *et al.*, 2003).

In this study, only 2/19 (10.52%) of common B-ALL patients expressed ZAP-70 as Calla positive ALL patients were significantly higher among the ZAP-70 negative patients especially children. Also, 2/19 (10.52%) of the pro-B-ALLs were positive for ZAP-70 expression. In contrast, Chiaretti *et al.* (2006) reported higher expression of ZAP-70 among the common B-ALL cases (52 and 50%) of pro-B cases, suggesting a maturative impairment that is often detected in acute leukemia and it is well established that the phenotypic repertoire of ALL blasts is never an exact replica of physiological expression regulation.

In the present study, 4/4 (100%) of the B-mature ALL cases expressed ZAP-70. Similar results were reported by Admirand *et al.* (2004), Sup *et al.* (2004), Carreras *et al.* (2005) and Chiaretti *et al.* (2006) suggesting that this gene could play a role in the pathogenesis of this disease. In Chiaretti *et al.* (2006) study, 2/2 (100%) of B-mature ALL patients expressed ZAP-70. Crespo *et al.* (2006) reported 4/6 (66%) positivity among the Burkitt's/ALL. Furthermore, the levels of ZAP-70 in these Burkitt's/ALL cases were found in the higher range of the series. The expression of ZAP-70 found in Burkitt's and in about 50% of CLL cases suggests that this gene is aberrantly expressed in mature B-cell derived neoplasms and that this gene could play a role in the pathogenesis of these diseases.

The patients received standard induction treatment. Comparing the treatment outcome of patients in respect to ZAP-70 expression, a significantly higher rate of adverse clinical outcome was found in ZAP-70 positive patients ($p = 0.003$). Among the negative group, (85.7%) achieved complete remission while (14.3%) had adverse clinical outcome (1 died, 2 resistant to induction). Among the ZAP-70 positive patients (36.84%) achieved complete remission while (63.32%) had adverse outcome (6 died, 6 resistant to induction). These data suggest that Zap-70 expression is associated with poorer outlook in B-cell-ALL patients. A statistically significant difference was noticed as regard the response to induction therapy among children ($p = 0.011$), while it did not differ among adults ($p = 0.085$).

This is in agreement with Chiaretti *et al.* (2006), who studied the mRNA expression of ZAP-70 and the clinical implication of its expression in both T and B-cells ALL and documented an association between higher expression of ZAP-70 with a shorter relapse free survival after achievement of complete remission, although they couldn't identify a cut-off value with strong statistical significant difference due to limited number of cases evaluated. Crespo *et al.* (2006) stated that there is no

correlation between ZAP-70 expression and the response to induction chemotherapy. This may be attributed to the exclusion of Burkitt's/ALL cases from their analysis.

Present results provide preliminary indication on the potential use of this protein as a prognostic marker also in ALL. Evaluation of a larger number of patients is needed to further validate this finding.

The discovery of tyrosine kinases that can be used as therapeutic targets is raising great interest. Since the introduction of STI-571 (imatinib), the outcome of patients with chronic myeloid leukemia (CML) and, probably, also of those suffering from BCR/ABL$^+$ ALL rearrangement, has improved (Rosti *et al*., 2004). These results clearly show that ZAP-70 may indeed be a therapeutic arget. ZAP-70 expression in a subset of patients with ALL opens the perspective of investigating the use of an inhibitor also in such cases.

CONCLUSION

In conclusion, results demonstrated that in B-lineage ALL, ZAP-70 expression increases along the maturation process of malignant B-cells and a high expression of ZAP-70 appears to correlate with an increased adverse response to chemotherapy especially in children. These results raise the possibility that ZAP-70 expression in B-cell ALL may have a prognostic value (being a bad prognostic marker), as well as being a candidate molecule for targeted therapies.

REFERENCES

Admirand, J.H., G.Z. Rassidakis, L.V. Abruzzo, J.R. Valbuena, D. Jones and L.J. Medeiros, 2004. Immunohistochemical detection of ZAP-70 in 341 cases of non-Hodgkin and *Hodgkin lymphoma*. Mod. Pathol., 17: 954-961.

Carreras, J., N. Villamor, L. Colomo, C. Moreno and S. Ramón y Cajal *et al*., 2005. Immunohistochemical analysis of ZAP-70 expression in B-cell lymphoid neoplasms. J. Pathol., 205: 507-513.

Chan, A.C., M. Iwashima, C.W. Turck and A. Weiss, 1992. ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. Cell, 71: 649-662.

Chan, A.C., D.M. Desai and A. Weiss, 1994. The role of protein tyrosine kinases and protein tyrosine phosphatases in T-cell antigen receptor signal transduction. Annu. Rev. Immunol., 12: 555-592.

Chen, Y.H., L.C. Peterson, D. Dittmann, A. Evens and S. Rosen *et al*., 2007. Comparative analysis of flow cytometric techniques in assessment of ZAP-70 expression in relation to IgVH mutational status in chronic lymphocytic leukemia. Am. J. Clin. Pathol., 127: 182-191.

Chiaretti, S., A. Guarini, M.S. De Propris, S. Tavolaro and S. Intoppa *et al*., 2006. ZAP-70 expression in acute lymphoblastic leukemia: Association with the E2A/PBX1 rearrangement and the pre-B stage of differentiation and prognostic implications. Blood, 107: 197-204.

Crespo, M., F. Bosch, N. Villamor, B. Bellosillo and D. Colomer *et al*., 2003. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N. Engl. J. Med., 348: 1764-1775.

Crespo, M., N. Villamor, E. Giné, A. Muntañola and D. Colomer *et al*., 2006. Z AP-70 expression in normal pro/pre B cells, mature B cells and in B-cell acute lymphoblastic leukemia. Clin. Cancer Res., 12: 726-734.

Cutrona, G., M. Colombo, S. Matis, D. Reverberi and M. Dono *et al*., 2006. B lymphocytes in humans express ZAP-70 when activated *in vivo*. Eur. J. Immunol., 36: 558-569.

Guillaume, N., C. Alleaume, D. Munfus, J.C. Capiod and G. Touati *et al*., 2005. ZAP-70 tyrosine kinase is constitutively expressed and phosphorylated in B-lineage acute lymphoblastic leukemia cells. Haematologica, 90: 899-905.

Marie, J.P. and O. Legrand, 1999. MDR1/P-GP expression as a prognostic factor in acute leukemias. Adv. Exp. Med. Biol., 457: 1-9 (Review).

Palacios, E.H. and A. Weiss, 2007. Distinct roles for Syk and ZAP-70 during early thymocyte development. The J. Exp. Med., 204: 1703-1715.

Rosti, G., G. Martinelli, S. Bassi, M. Amabile and E. Trabacchi *et al*., 2004. Molecular response to imatinib in late chronic-phase chronic myeloid leukemia. Blood, 103: 2284-2290.

Schweighoffer, E., L. Vanes, A. Mathiot, T. Nakamura and V.L. Tybulewicz, 2003. Unexpected requirement for ZAP-70 in pre-B cell development and allelic exclusion. Immunity, 18: 523-533.

Sup, S.J., R. Domiati-Saad, T.W. Kelley, R. Steinle, X. Zhao and E.D. His, 2004. ZAP-70 expression in B-cell hematologic malignancy is not limited to CLL/SLL. Am. J. Clin. Pathol., 122: 582-587.

Verhoeyen, E., V. Dardalhon, O. Ducrey-Rundquist, D. Trono, N. Taylor and F.L. Cosset, 2003. IL-7 surface-engineered lentiviral vectors promote survival and efficient gene transfer in resting primary T lymphocytes. Blood, 101: 2167-2174.

Wiemels, J.L., R.N. Smith, G.M. Taylor, O.B. Eden and F.E. Alexander *et al*., 2001. Methylentetrahydrofolate reductase polymorphism and the risk of molecularly defined subtypes of childhood acute leukemia. Proc. Natl. Acad. Sci. USA., 98: 4004-4009.