Original Articles/นิพนธ์ต้นฉบับ

ZAP-70 Expression and Its Prognostic Significance in Childhood B-lineage Acute Lymphoblastic Leukemia.

Paisooksantivatana K[1], Iamsa-art C[1], Rungsimaporn B[2], Kanjanapongkul S[3], Worapongpaiboon S[4], Pakakasama S[5]

[1] Flow Cytometry Unit and [2] Human Genetics Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

[3] Hematology Unit, Department of Pediatrics, Queen Sirikit National Institute of Child Health, Bangkok, Thailand

[4] Pathology Unit, Samitivej Hospital, Bangkok, Thailand

[5] Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Abstract

Background: ZAP-70 expression was found in normal human B cell precursors and B lymphoproliferative disorders. Since the level of ZAP-70 expression is prognositic in chronic lymphocytic leukemia, a similar role may be demonstrable in other B-lineage malignancies. The present study investigates the expression of ZAP-70 in B-lineage acute lymphoblastic leukemia and its prognostic significance.

Methods: Thirty-three bone marrow and blood samples were obtained from children newly diagnosed with B-lineage ALL. ZAP-70 was determined using flow cytometry. Correlation between expression of ZAP-70 and clinicobiological presenting features (age, sex, white blood cells) and cytogenetics was performed.

Results: ZAP-70 was expressed in all cases with a wide variation (0.11 - 87.68%). There is no correlation between ZAP-70 expression level and clinicobiological presenting features also the cytogenetics abnormalities was found. Aberrantly high expression of ZAP-70 was found in three cases, two of which had aberrant CD13 and CD33 co-expression.

Conclusions: Aberrantly high expression of ZAP-70 might be associated with certain subgroup (CD13 and CD33 coexpression) of B-lineage ALL.

Keywords: ZAP-70; Acute lymphoblastic leukemia

Corresponding author: Paisooksantivatana K, MD.
Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand Tel : 66 2 2011436 Fax : 66 2 2011150
E-mail address : rakpz@mahidol.ac.th

Introduction

ZAP-70 (Zeta-associated-protein 70 kDa), which is a protein tyrosine kinase (PTK) made up of 619 amino acids, is involved in proximal T-cell receptor (TCR) signaling. Activation of TCR results in phosphorylation of tyrosine and TCR-associated ZAP-70, which in turn causes activation of tyrosine kinase activity[1]. ZAP-70 is crucial for T-cell signaling and development as mutation or absence of ZAP-70 results in a severe immunodeficiency characterized by a deficit of peripheral blood CD8 cells and dysfunctioning CD4 cells[2]. ZAP-70 was initially identified in T cells and NK cells and was once thought to be limited to these lineages until expression of ZAP-70 during B-cell development was demonstrated in murine model where it appears to play a role in the transition of pro-B to pre-B cell in bone marrow[3].

It is more interesting since it has been shown that a subset of cases of chronic lymphocytic leukemia (CLL) expresses ZAP-70[4]. Moreover, it has a prognostic significance which is stronger than any biological factors previously demonstrated[5-9]. In addition, expression of ZAP-70 in normal B cells and many kinds of B-cell malignancy have been reported[10,11]. Since ZAP-70 has the important prognostic significance in B-CLL, this role may exist in other B lymphoproliferative disorders. Therefore, we have determined ZAP-70 expression in B-lineage acute lymphoblastic leukemia (ALL) and its prognostic role as compared with clinicobiologic features and minimal residual disease (MRD).

Materials and Methods

Samples

The study samples included six peripheral blood and twenty-seven bone marrow samples from children who were newly diagnosed with B-lineage ALL before treatment with chemotherapeutic agents. Diagnostic immunophenotyping was performed in all cases by standard techniques[12] and interpreted by a well-qualified hematopathologist. Cytogenetics studies were done in six cases using standard G-banding with trypsin-Giemsa staining[13]. After diagnostic immunphenotyping was completely performed, each sample was immediately processed for maturation stage and ZAP-70 determination within 24 hours of collection.

Maturation status assessment and evaluation of ZAP-70 expression

Flow cytometry analysis of ZAP-70 was done as previously described[14,15] on different cell subsets according to their maturation status. B-lineage leukemic cells were characterized for the expression of surface and cytoplasmic markers using monoclonal antibodies to surface markers including CD19 Tricolor (Caltag/Invitrogen), CD10 PE (Immunotech), $F(ab')_2$ anti-human IgM FITC (DAKO) and $F(ab')_2$ isotype control (DAKO). Monoclonal antibodies to cytoplasmic markers included ZAP-70 Alexa-488 (Caltag/Invitrogen), IgG1 isotype Alexa-488 (Caltag/Invitrogen), $F(ab')_2$ anti-human IgM FITC (DAKO) and $F(ab')_2$ isotype control (DAKO). Briefly, the staining procedure was performed as follow: one million leukemic cells were incubated with recommended amount of monoclonal antibodies to surface markers in a dark at room temperature for 20 minutes. Then red blood cells lysis was done by 20-minute incubation with 2 ml FACS Lysing reagents (Beckton Dickinson). The sample tubes that need no further intracellular staining were washed twice and resuspended in 0.5 mL of phosphate buffer saline (PBS) with 0.5% bovine serum albumin and 0.02% NaN_3 and 0.5% paraformaldehyde before being kept in dark at 4°C while waiting for acquisition. For intracellular staining, after surface staining and red blood cell lysis, leukemic cells were incubated with 0.5 mL FACSPermeabilizing reagent (Beckton Dickinson) for 15 minutes and washed. Recommended amount of monoclonal antibody for intracellular markers were added and incubated for 10 minutes at room temperature. Then cells were washed twice and

resuspended in PBS (with 0.5% bovine serum albumin and 0.02% NaN_3) and 0.5% paraformaldehyde. Afterwards, all samples were analyzed by FACScan flow cytometer (Beckton Dickinson, San Jose, CA). One hundred thousand cells were acquired for each tube. List mode data files were analyzed using CellQuestPro Program (Beckton Dickinson, San Jose, CA). Samples were classified according to their maturation status by expression of surface CD19, CD10, cytoplasmic IgM (cIg) and surface IgM (sIg) as follow: early pre-B (CD19+/CD10-/cIg-), common-B (CD19+10+cIg-) and pre-B (CD19+10+cIg+)[16]. Gating strategy for ZAP-70 analysis was shown in Figure 1.

Risk classification

Patients were considered to have low-risk ALL if they were 1 to 9 years old and a presenting leukocyte count lower than 50,000 cells/mm^3. Other patients without the just-mentioned features were considered high-risk. Cytogenetics abnormality was not included in risk classification criteria since the results were available only in some cases.

Statistical analysis

Difference of ZAP-70 expression in the each clinicobiological presenting feature and risk group was compared using Mann-Whitney U test. The difference was determined as statistically significant if $p<0.05$.

Results

Thirty-three children with a median age of 4 years (range, 7 months to 17 years) were enrolled in this study. The initial presenting features of all patients are shown in Table 1. With available data, we could

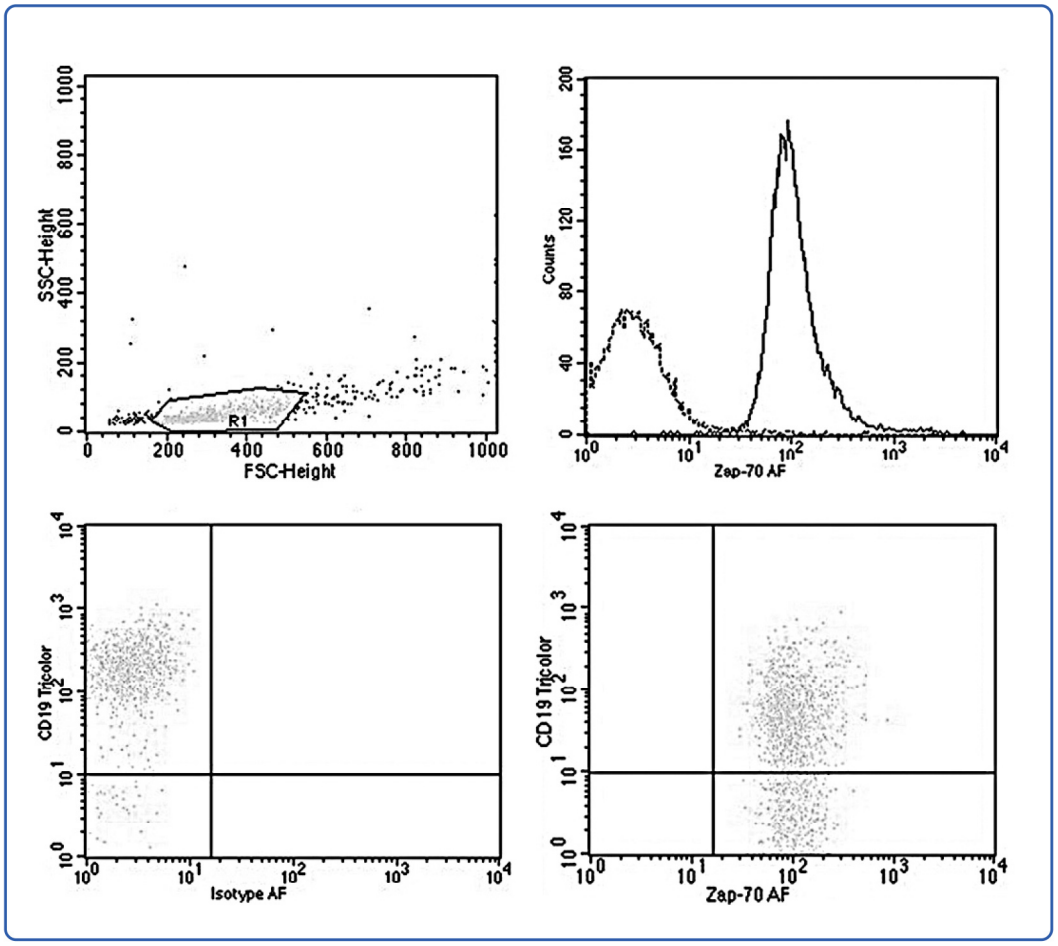

Fig.1 *Gating strategy for ZAP-70 determination (AF = AlexaFluor488)*

Table 1 Clinicobiological presenting features of patients.

Case	Age (years)	Sex	WBC (K/μL)	Maturation status	Aberrant expression
ALL 1	17	m	18.3	common-B	No
ALL 2	13	m	149.0	pre-B	No
ALL 3	15	f	25.2	pre-B	No
ALL 4	2	m	13.6	pre-B	No
ALL 5	5	m	109.0	pre-B	No
ALL 6	2	m	476.0	pre-B	No
ALL 7	14	m	6.1	pre-B	No
ALL 8	4	m	4.9	common-B	No
ALL 9	2	f	85.2	pre-B	No
ALL 10	8	f	178.3	pre-B	No
ALL 11	1	m	28.0	pre-B	No
ALL 12	9	f	52.0	common-B	CD13,33
ALL 13	3	m	28.0	pre-B	No
ALL 14	1	f	N/A	pre-B	No
ALL 15	2	m	7.9	pre-B	No
ALL 16	2	m	4.7	pre-B	No
ALL 17	8	m	56.4	pre-B	No
ALL 18	3	m	60.0	pre-B	No
ALL 19	7	f	26.4	common-B	No
ALL 20	7 months	f	108.6	pre-B	No
ALL 21	2	m	N/A	pre-B	No
ALL 22	2	m	20.5	pre-B	No
ALL 23	12	m	N/A	pre-B	CD13
ALL 24	4	f	14.1	pre-B	No
ALL 25	1	f	N/A	pre-B	CD13,33
ALL 26	10	f	143.3	pre-B	CD13,33
ALL 27	9	f	N/A	common-B	CD33
ALL 28	8	f	20.3	pre-B	CD13
ALL 29	2	f	1.2	pre-B	No
ALL 30	4	m	N/A	pre-B	No
ALL 31	4	m	1.5	pre-B	No
ALL 32	11	m	26.9	pre-B	CD14
ALL 33	2	f	1.6	pre-B	No

classify 33 patients into low risk (n= 15) and high risk (n= 18). Pre-B (n=28) was the most common stage of B-lineage ALL followed by common-B (n=5). Seven cases had cross-lineage aberrant expression of cell surface markers including six cases with expression of CD13 and/or 33 and one case with expression of CD14. The cytogenetics result was available in 6 cases. Three of six had normal karyotype. Other three had BCR-ABL (ALL3), complex chromosomal abnormalities (ALL28) and hyperdiploidy (ALL29).

ZAP-70 was expressed in leukemic cells of all cases. The level of ZAP-70 was varied from 0.11-87.68% (Figure 2). The percentage of ZAP-70 expression was not different among each clinicobiological presenting features (age, sex, white blood cell, and risk group) also the type of samples as shown in Table 2. The expression level of ZAP-70 was not different in each maturation status of B cell. Interestingly, two of three cases with co-expression of CD13 and CD33 had very high levels of ZAP-70 expression (Figure 2).

Discussion

ZAP-70 is a PTK involved in proximal TCR signaling ZAP-70 which is crucial for T-cell signaling and development (1). ZAP-70 was once thought to be exclusive to T/NK cell lineage; however, expression of ZAP-70 during B-cell development was also

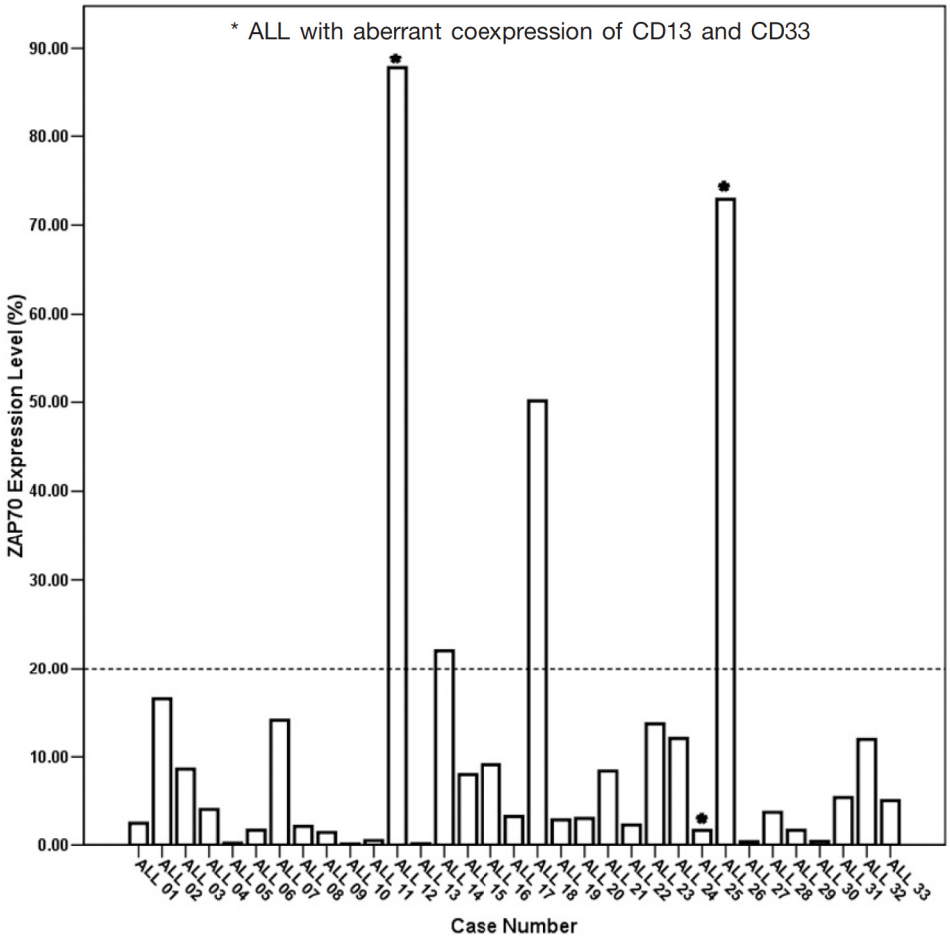

Fig.2 *There were thirty three samples in this study. Six cases (ALL7, ALL13, ALL22, ALL24, ALL27 and ALL31) were peripheral blood, otherwise were bone marrow. Three cases had abnormal cytogenetics (ALL3 with BCR-ABL, ALL28 with complex karyotypes and ALL29 with hyperdiploidy). ZAP-70 was variably expressed in each case, ranging from 0.11% to 87.68%.*

Table 2 There is no correlation between ZAP-70 expression and clinicobiological presenting features also a type of samples.

Presenting features	n	Zap-70 expression (%, mean ± SD)	p-value
Age, y			0.067
Younger than 1	1	2.96	
1 to 9	25	9.36 ± 19.35	
More than 9	7	19.68 ± 24.07	
Sex			0.872
Male	19	7.98 ± 11.52	
Female	14	15.92 ± 28.03	
WBC, x 10^9/L			0.725
Less than 50	17	5.38 ± 4.49	
50 or more	10	23.67 ± 33.67	
Maturation stage of B-cell			0.379
Common B	5	18.58 ± 38.64	
Pre B	28	10.06 ± 15.91	
Risk group			0.315
High risk	18	4.98 ± 5.48	
Low risk	15	19.01 ± 28.02	
Type of samples			0.744
Blood	6	5.73 ± 6.04	
Bone marrow	27	12.60 ± 22.10	

demonstrated in murine model where it appears to play a role in the transition of pro-B to pre-B cells in bone marrow[3]. Since then expression of ZAP-70 in human normal and malignant B cells was further demonstrated by many methods such as immuno-histochemistry, flow cytometry and PCR[10,11,14,17,18]. The low level expression of ZAP-70 compared to T/NK lineage is found in normal human B lymphocytes expressing ZAP-70 in every maturation stage at different levels depending on their differentiation, activation and tissue localization[10,14,17]. The highest level of ZAP-70 in normal B-lineage determined by flow cytometry, reported by Scielzo et. al., is 27.3%[10]. In addition, ZAP-70 expression can be modulated following stimulation via the B-cell receptor which indicates the potential role of ZAP-70 in the signaling pathway of B lymphocytes at different maturation stages[10].

ZAP-70 expression has shown prognostic significance in patients with CLL[5-9]. The higher ZAP-70 level correlates with unmutated status of IgVH gene and poor prognosis. The role of ZAP-70 in biopathobiology of CLL is not clearly known. The one possible explanation is that ZAP-70 level may reflect

the in vivo activation status[10,19]. Since the expression of ZAP-70 in CLL correlates with the pathobiology of disease and has a strong prognostic value, such findings would be expected in other B cell malignancies.

ZAP-70 expression in B-lineage ALL has been demonstrated with different techniques including immunohistochemistry, immunofluorescence, Western blot, flow cytometry, and reverse transcription-PCR[10,11,14,18,20,21]. In those reports, ZAP-70 was detected in 29% to 100% of B lineage ALL cases with heterogeneous expression. The expression of ZAP-70 was confirmed in the present study in which ZAP-70 was detected by flow cytometry in all thirty-three cases of childhood B-lineage ALL with common-B and pre-B phenotypes. Levels of expression were found to vary from 0.11% to 87.68%. The possibility of using ZAP-70 as a prognostic factor in B-lineage ALL has been proposed[18]. Chiaretti et al. (2006) reported the association of high level of ZAP-70 and the E2A/PBX1 rearrangement. Furthermore, longer disease-free survival rate was found in B-lineage ALL cases bearing no molecular abnormalities and lower level of ZAP-70[18]. In the present study we found no correlation between level of ZAP-70 expression and either clinicobiological presenting features (age and white blood cells) or cytogenetics results. There was no difference of level of ZAP-70 expression between a case with favorable cytogenetics (ALL29 with hyperploidy) and another one with unfavorable cytogenetics (ALL3 with BCR-ABL). Since ZAP-70 was constitutionally expressed at a low level in normal B cell lineage, the aberrantly high expression of ZAP-70 in B-lineage ALL may be of some interest. Our data demonstrated that two of three cases which expressed highest level of ZAP-70 expression had aberrant CD13 and CD33 co-expression. This was of interest since aberrant expression of myeloid marker in ALL portends a poor prognosis and poor response to conventional drug therapies[22].

Low level expression of ZAP-70 is essential for maturation of normal B cell precursors but aberrantly high expressed in B lineage ALL may play an important role in leukemogenesis in subpopulation of cases and may be useful for prognostic determination. However, a further sophisticated and larger study is required to understand the role of ZAP-70 in B-lineage ALL as well as other B cell lymphoproliferative disorders.

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