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Zeta Chain-Associated Protein-70 Expression is a Prognostic Tool in Mature and Precursor B-Cell Neoplasm

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Abstract: Problem statement: The Zeta Chain-Associated Protein (ZAP) is a 70-kDa molecule associated with the zeta chain of the CD3 receptor complex of the lymphocyte population. It plays an important role in signaling initiation, activation T-cell receptor, phosphorylation of multiple downstream targets. So that believed as T. lymphocyte specific .Several studies is gathering that the pattern of expression is not lineage specific. We aimed to study the role of ZAP-70 /CD38 as new prognostic marker in CLL and its prognostic value in ALL patients. **Approach:** Our study included 49 ALL ranged from 2-42 years they were 27 males and 22 females. One hundred fifty six patients with CLL. They were 87 males and 69 females, their ages ranged from 50-77 yearsImmunophenotyping. by flow cytometry (EPICS XL-2and 4 color Bechman Coulter USA), intracellular ZAP70 protein was assessed by flow cytometry using (Caltag laboratories, Beckman Coulter USA). **Results:** Immunophenotyping of 49 ALL patients ZAP-70 was found at significantly higher levels in T-ALL cases compared with B-lineage ($p<0.001$), however, a proportion of B- lineage ALL showed high levels of ZAP-70. Moreover we observed that ZAP-70 is higher in pre-B ALL than pro B ALL and common ALL with more decline in mature B-ALL. Follow up patient by zap, pretreatment versus post treatment $p = 0.008$, correlation of zap with hematological and clinical parameter in ALL, no significant correlation except positively correlated with leucocytic count while in CLL highly significant correlation with splenomegally but not with lymphadenopathy, strong association between zap-70 and binnet staging but not with CD38. Overall survival of zap-70 in ALL/CLL show that short free survival associated with positive zap-70. ZAP-70 has raised great interest in CLL patients because it represents a powerful prognostic marker. **Conclusion:** Our results provide preliminary indication on the potential use of this protein as a prognostic marker also in ALL. These results clearly show that ZAP-70 may indeed be a therapeutic target. ZAP-70 expression in a subset of patients with ALL opens the perspective of investigating the use of an inhibitor and can be a candidate molecule for targeted therapy.

Key words: ZAP-70, cell neoplasm, Zeta Chain-Associated Protein (ZAP), T-Cell Receptor (TCR), therapeutic target, binnet staging, powerful prognostic, positive zap, bechman coulter

INTRODUCTION

The Zeta Chain-Associated Protein (ZAP) is a 70-kDa molecule associated with the zeta chain of the CD3 receptor complex of the lymphocyte population (Chan *et al*., 1992) and belongs to the Syk (spleen tyrosine kinas) family of tyrosine kinesis. ZAP-70 plays an important role in signaling initiation, activation and phosphorylation of multiple downstream targets. Briefly, after T-Cell Receptor (TCR) ligation, activation of Src kinase occurs and induces activation of the Immune receptor Tyrosine-based Activation Motifs (ITAMs) (Kane *et al*., 2000). This process eventually leads to the recruitment and activation of ZAP-70, which in turn activates several downstream targets, such as phospholipase $C\gamma/Ca^{++}$ signaling pathway and the Mitogen-Activated Protein Kinas (MAPK) pathway (Qian and Weiss, 1997).

A similar pathway is sustained in B cells by Syk: activation of the B-Cell Receptor (BCR) induces rapid phosphorylation of ITAMs of Igα and Igβ. Once phosphorylated, ITAMs recruit Syk, thus inducing its phosphorylation and the activation of several downstream targets. Interestingly, ZAP-70 was

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originally described to be exclusively present in T cells and natural killer cells but several studies is gathering that the pattern of expression of ZAP-70 might not be a lineage specific as previously thought (Scielzo *et al.*, 2006).

The role of ZAP-70 molecule in B-cell development has been recently established in a mouse model. Who has shown that this protein is also expressed in B-lineage cells and that it appear to play a role in their early development and in particular during in pro-B to pre-B transition stage. (Schweighoffer *et al*., 2003) Variability of CLL in clinical course and prognosis is a point of research of several studies. Some patients are discovered accidentally and show an indolent disease and never require treatment. However, a substantial number of patients die of aggressive disease and require intensive treatment shortly after diagnosis (Kipps, 2000) The traditional staging systems devised by Rai and Binet (Rai *et al*., 1975; Binet *et al*., 1981) are useful methods of assessing the survival and the treatment requirements in B-CLL patients. However, these systems are of limited prognostic value in the early stages of the disease which include most of patients at diagnosis. Thus, there is a need to seek out other prognostic factors in the early stage of the disease to identify stable or progressive forms of CLL that might facilitate risk-adapted treatment strategies (Byrd *et al*., 2000). Therefore, a number of studies have focused on identifying novel prognostic markers, which may help better survival rate.

Genetic study of somatic mutations in the immunoglobulin heavy-chain variable region (IgVH) of B-CLL cells has been described as one of the most powerful prognostic factors distinguishing two disease subsets (Hamblin *et al*., 1999). The cases with mutated IgVH genes exhibit a favourable clinical course and they may never require treatment while patients with unmutated IgVH genes are characterized by a reduced survival and responsiveness to chemotherapy. However, IgVH sequencing is difficult to perform in a routine diagnostic laboratory. Finding a surrogate for IgVH mutational status would seem to be an important priority (Oscier *et al*., 1997).

Schweighaffer 2003 studies the role of ZAP-70 and Syk in the early development during the pro B-ALL to pre B transition stage. Their data demonstrated functional redundancy between there 2 kinases and also showed that the absence of both kinases and not only Syk is necessary to block pre-BCR induced events such as differentiation into pre B cells, cell proliferation and heavy chain allelic exclusion (Schweighoffer *et al*., 2003). The objective of our study was to assess the impact of ZAP-70/ CD38 on CLL and to evaluate prognostic significance of ZAP -70 in B-ALL patients.

MATERIALS AND METHODS

Subject and method: Two groups of patients were included in our study. They were recruited from Oncology Center, Mansoura University and gave them their informed consent.

Group-I comprised: Forty nine ALL patients. Their ages ranged from 2-42 years. They were 27 males and 22 females. The diagnosis was based on morphological, cytochemical, immunophenotyping. Bone marrow aspirate or peripheral blood were immediately transported in Na heparin tubes to Flowcytometry laboratory Mononuclear cells were stained with fluorochrome-conjugated monoclonal antibodies and stained with the following combination: Fluroscein Isothiocyanate (FITC), Phycoerythrin (PE) and Phycoerythrin-Cyanine 5(PC5) labeled monoclonal antibodies against the following antigens and analyzed by flow cytometry(EPICS XL-2and 4 color Bechman Coulter USA). B-lineage leukemia cells were characterized by the surface expression of CD19, CD20, CD22, CD10, (monoclonal mouse antihuman Dako .Denmark). After fixation and permeabilizaion, leukemic cells were evaluated for the intracytoplasmic and nuclear expressioin of CD79a, IgM and TdT (Bechaman coulter, immunotech, France).

Group-II: One hundred fifty six patients with CLL. They were 87 males and 69 females, their ages ranged from 50-77 years. Diagnosis based on morphological, bone marrow aspiration, immunophenotypic criteria. The following monoclonal antibodies were assessed by flow cytometry: CD19, CD5, CD23, CD20, Ig Kappa (IgK) and lambda (Ig λ) light chains. Additional prognostic markers were used including Zap70 and CD38.

For determination of intracellular ZAP70 protein, the lysed whole blood cell were fixed and permealized with fix and perm kit (Caltag laboratories, Beckman Coulter USA). 10 μL of PE labeled mouse anti-ZAP70 monoclonal antibodies were added followed by 20 min incubation at room temperature in the dark. After two washings with PBS, 10 μL of PC-5 labeled anti CD19 and FITC labeled anti CD5 were added and reincubated for 15 min at room temperature. Corresponding negative isotype matched controls were simultaneously prepared. Analysis was done for determination of CD5/CD19/ZAP70 positive cells by gating CD19

positive lymphocytes which express both CD5 and ZAP70. Samples expressing ZAP70 by more than 30% were considered positive.

For determination of CD38 marker, Flurescein Isothiocyanate (FITC)-labeled monoclonal mouse anti-human CD38 FITC (Clon AT 13/5. Dako) were used.

An acquistation gate was established based on FSC and SSC that included mononuclear cells and excluded dead cells and debris. 10.000 events were collected and analyzed. Each sample was run with an appropriate isotopic control that was used to define the negatively stained cell.

Followed up patients with CLL were done for 2 years, while those with ALL were followed up for one year.

Statistical analysis: The statistical analysis of data done by using excel program and SPSS program statistical package for social science version 10. The description of the data done in form of mean (+/-) SD for quantitative data. The analysis of the data was done to test statistical significant difference between groups.

To test association between variable correlation co-efficiency test was used N.B: P is significant if < or = 0.05 at confidence interval 95%.

RESULTS

All group: Our study on ALL cases are presented with cervical lymphadenopathy only 59% while other with generalized lymphadenopathy 41%, some cases presented with splenomegally 33% while 20% with hepatomegally and splenomegally. Morphological examination of peripheral smear show infiltration by immature mononuclear cells with large nucleo cytoplasmic ratio, scanty cytoplasm, fine immature chromatin with 1-2 indistinct nucleoli. The descriptive clinical and hematological data of studied groups are illustrated in Table 1.

Immunophenotyping of 49 ALL patients: Thirteen cases were T ALL and 36 cases were represented by B-lineage ALL.

Within the B- lineage group further immunophenotyping characterization showed, 16/49 were pre-B stage, 10/49 were considered as common B-ALL (CD10) and 8/49 were pro B-ALL. ZAP-70 was found at significantly higher levels in T-ALL cases compared with B-lineage ($p<0.001$), however, a proportion of B- lineage ALL showed high levels of ZAP-70. Moreover we observed that ZAP-70 is higher in pre-B ALL than pro B ALL and common ALL with more decline in mature B-ALL as shown in Table 2.

Table 1: Descriptive clinical and hematological data of ALL and CLL patients

	ALL N=49	CLL N=156
Age (years)	9.88 ±7.82 (2-42)	63.42 ± 6.76 (50-77)
Sex		
♂	27/49	87/156
♀	22/49	69/156
Hb(g/dL)	8.52 ± 2.96 (3.4-12.6)	10.13 ± 1.29 (7.5-13.5)
WBCs ($\times10^9$ L^{-1})	23.37 ± 12.03 (5.5-52.7)	68.2 ± 34.15 (28.156)
Platelet ($\times10^9$ L^{-1})	95.39 ± 70.21 (16-273)	130.98 ± 38.12 (57-212)
Peripheral blast	22.45 ± 9.21 (10-55)	----------
Bone marrow blast%	71.35 ± 18.76 (36-97)	————
LN ≥3	20 (41%)	108 (69%)
<3	29 (59%)	48 (31%)
Spleen	16 (33%)	54 (34.6%)
Liver	10 (20%)	18 (12.5%)

The data were presented as Mean ± SD

Table 2: ZAP 70 according to immunphenotyping at diagnosis (n= 49)

Immunophenotyping	No	Mean ± SD	Range	P- value
T-ALL	13	72.0±10.8	49-83	0.000
Pre-B	16	28.0±13.8	34-78	
Common	10	35.4±6.4	39-62	
Pro-B	8	44.0±11.5	33-63	
Mature	2	7.2±1.8	6-11	

The data were presented as Mean ± SD

Table 3: Wilcox on singed rank test for ZAP-70 level in the 49 ALL patients investigated before and after induction chemotherapy and after l year of follow up

ZAP-70 (%)	At diagnosis (before treatment) (n= 49)	After one month (n=45)	After one year (n = 43)
Mean ± SD	55.64 ± 15.39	35.02 ± 15.82	18.34 ± 11.66
Median	55.00	36.00	14.00
Range	33-83	3-63	6-47

Pre treatment versus post treatment ($p = 0.008$)

Table 4: Correlation of ZAP -70 at diagnosis with hematological and clinical parameter in ALL

	r	p
Age (years)	-0.187	0.208
Sex	-0.146	0.328
Hb	-0.111	0.459
TLC	0.249	0.045*
Platelet	-0.064	0.671
Peripheral-blast	-0.017	0.910
Bone marrow blast	-0.138	0.354
FAB	0.044	0.770
L.N	0.040	0.787
Spleen	-0.230	0.120
Liver	-0.180	0.225

After induction and maintaince protocol therapy follow up ALL patients for one year, we observed a decline of ZAP-70 expression as demonstrated at Table 3.

The correlation studies revealed a significant positive correlation between ZAP-70 expression and total leukocyte count ($p = 0.045$) with no significant correlation with other studied parameters Table 4.

Table 5: Correlation of ZAP 70 with clinical parameters in CLL group

		ZAP-70 expression		
		+ ve	- ve	P-value
Spleen	-	36	21	0.001
	+	48	23	
	++	20	8	
Liver	-	24	33	
				0.008
	+	62	37	
LN	-	32	-	
				0.235
	+	72	11	
	++	45	16	

Table 6: Correlation of ZAP -70 and CD38 with clinical Binnet staging

	Stage A	Stage B	Stage C	P-value
ZAP –ve (n = 59)	59	-	-	0.005
ZAP +ve (n= 97)	23	46	28	
CD38 -ve	82	33	14	0.879
CD38 +ve	16	10	3	

Table 7: correlation of ZAP 70 with CD38

	ZAP expression		
	+ ve	- ve	P- value
CD38 - ve	74	83	0.974
CD38 +ve	82	73	
Total	156	156	

Table 8: Multivariate regression analysis of ZAP 70 with other variants

	95% confidence interval for B			
Model	Lower bound	Upper bound	t	p
ZAP 70	-0.458	-0.0130	-2.218	0.039*
Liver	-0.318	0.1560	-0.689	0.494
WBCs	-0.006	-0.0010	-2.475	0.017*
CD38	-0.200	0.0167	-0.177	0.860
LDH	0.000	0.0010	0.726	0.471
Peripheral blast %	-0.314	0.2650	-0.837	0.482
Bone marrow blast %	0.000	0.0030	0.568	0.368

Zap-70 was not significantly correlated with age, sex and hematological parameters except total leucocytic count which was significantly positively correlated with Zap-70 expression (p = 0.045).

CLL group: Our study on 156 patients presented with variable leucocytosis (28-156 × 10^3 μL^{-1} and presented with lymphadenopathy , splenomegally and sometimes hepatomegally as shown in Table 1.

Table 5 illustrates the relation between ZAP70 expression with some clinical parameters in CLL group. The ZAP70 was highly expressed in patients with splenomegaly and hepatomegaly (p = 0.001 and 0.000 respectively). On the other hand, the ZAP70 expression was not affected by the number of LN enlargement (p = 0.235).

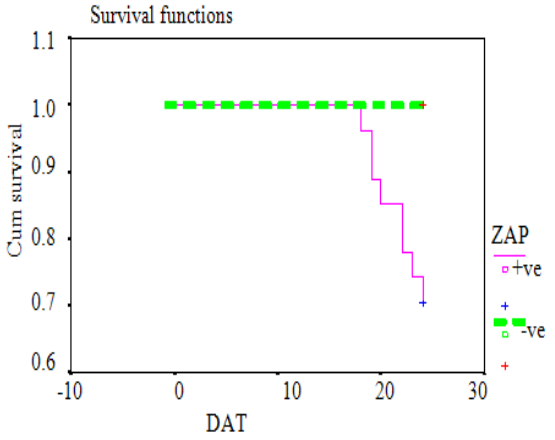

Fig. 1: Treatment-free interval in CLL patients was calculated from time of diagnosis using Kaplan - Meier plots, we observed that ZAP 70 +ve patients had short free survival than ZAP-ve patients

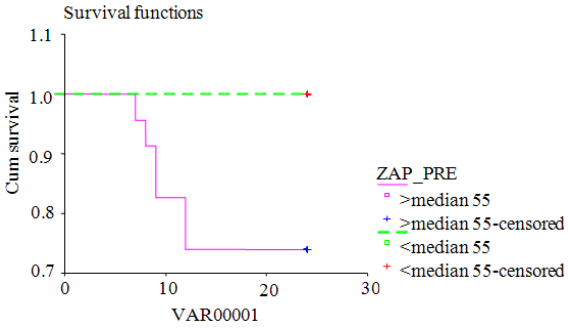

Fig. 2: Kaplan-Meier plots for treatment– free interval in ALL patients was calculated from time of diagnosis. We found that ZAP 70 +ve median >55 as cut off had short free survival than ZAP - ve median <55 that carry unfavorable prognosis

Expression of ZAP70 and CD38 in relation to Binnet staging is shown in Table 6. There is a strong association between ZAP70 expression and late stages. In contrast, no significant association was encountered between CD38 expression and the clinical stage (p = 0.879). Moreover, no significant correlation was found between ZAP70 and CD38 expression in CLL group Table 7. Multivariate regression analysis of ZAP 70 with other variants were shown in Table 8.

Overall survival in CLL patients and ALL patients with ZAP70 positive and negative patients were shown in Fig. 1-2.

DISCUSSION

ZAP-70 is a tyrosine kinase previously known to be T-lymphocyte specific and responsible for T cell signaling and activation. In our present study we observed high expression of ZAP-70 in a mature B-cell derived neoplasm as CLL where it seems to contribute to enhancing signal from the BCR (Chen *et al*., 2002). It was also found in other hematological malignancies as mature cell lymphoma, diffuse large B-cell lymphoma and Burkitts lymphoma (Sup *et al*., 2004; Admirand *et al*., 2004).

These results pose the question where is ZAP-70 expression in CLL is an aberrant phenomena related to the neoplasm. The mechanism of abnormal expression of ZAP-70 in CLL are not fully understood, it has been hypothesized that the expression of ZAP-70 was derived from the cell that leukemia originated from, or that its expression was ectopic in this lineage. Recently, ZAP-70 has been shown to be expressed in mice pro/pre B cells (Schweighoffer *et al*., 2003), its presence being important for B- cell development. Based on previous studies prompted us to analyze ZAP-70 expression in different B-ALL subsets. We found high ZAP-70 expression in pre B-ALL than pro B-ALL and decline towards common ALL(CD10+). This result was in concordance with (Yeoh *et al*,, 2002), who found ZAP-70 expression in pre B-ALL at proteins levels and m RNA, also with Wandroo *et al*. (2008) who do study in quantitative PCR assay and found ZAP-70 expression was strongly expressed in 9/12 cases of primary B-cell lymphoblastic leukemia, our data clearly indicate that ZAP-70 expression increases along with the differentiation and maturation process of B-cell population. This agree with Chiaretti *et al*. (2006). ZAP70 is preferentially expressed in cases carrying the E2A/PBX1 rearrangement indicating that ZAP70 expression increases along with the differentiation and maturation process of the B-cell population. These proteins are probably acting as functionally redundant signaling molecules to the pre-BCR components (Oya *et al*., 2003). These results strengthen the data previously reported in mice, indicating that ZAP70 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-BCR-induced events, such as differentiation into pre-B cells, cell proliferation and heavy chain allelic exclusion (Schweighoffer *et al*., 2003).

In the study of Wandroo *et al*. (2008) ZAP-70 highly expressed in pre-B lineage cells and most cases of pre-B acute lymphoblastic leukemia. This may hold prognostic value for pre-B ALL and raises the prospect of a novel therapeutic target, while Crespo (Chiaretti *et al*., 2006) found no relation between ZAP-70 expression levels and the maturation status of the B-ALL or the detected genetic abnormalities .

Overall, we found a high expression of ZAP-70 in 24 of 49 (49%) B-ALL pro/pre phenotype. Moreover, 2of 49 Burkitt /ALL showed lower level of ZAP-70 expression. This was not in accordance with Crespo *et al*., (2006) who found higher ZAP-70 expression in these Burkitt /ALL cases while Wandroo *et al*. (2008) found that ZAP-70 expression was not detected in mature B cell lines. This may be due to low number in our study and different methodology.

ZAP-70 is highly expressed in B-ALL before induction, when followed up for one year, then gradual decline after one month, with more regression after maintaince therapy (p=0.008 pretreatment vs post treatment). This is in agreement with Crespo *et al*., (2006), who found no correlation between ZAP-70 expression and response to the induction treatment .This may explained by nature of patients, protocol of therapy and different methodology.

Correlation of CD38 with ZAP 70 show no significant correlation (p 0.974). this is in agreement with chantepie *et al*. (2010) who found significant correlation ($p< 0.001$) by pyrosequencing method. This discrepancy due to different methodology. CD38 expression by flowcytometry with ZAP 70 giving different panel. Correlation of CD38 with clinical Binnet staging revealed no significant difference. This finding did not agree with Domingo-Domenech *et al*. (2002) while ZAP 70 expression highly significant with binnet staging $p<0.005$, this agree with Gachard *et al*. (2008) who found ZAP 70 was significantly expressed in 28, 54 and 61% of patient with Binet stage A, B, C B cell CLL respectively $p = 0.008$ .Treatment-free survival of ZAP-70 +ve /CD38 +ve giving short free survival than ZAP-ve /CD38+ve indicating that combined two variable asses more prognosis, more stratification, better management. In the multivariate regression analysis, ZAP 70 is only prognostic marker while CD38 lost statistical significance. This agrees with Crespo *et al*., (2003).

The molecular mechanism underlying aberrant ZAP-70 expression in B-CLL cells and its functional implication for biological behavior of the malignant cell clone are currently unknown. Chen *et al*. (2002) suggested that BCR stimulation in CLL cells results in tyrosin phosphorylation and translocation of ZAP-70 to the plasma membrane that associated with surface immunoglobulin and CD79b. Furthermore, recent studies have shown that ZAP-70 is tyrosine

phosphorylated in response to antibody mediated CD38 stimulation in NK &T cell raising the possibility that it play a role in CD38 singling in B-CLL (Deaglio *et al*., 2002; Zubiaur *et al*., 2002).

Our data indicate that ZAP70 expression correlates with a shorter relapse-free survival after achievement of CR, although a cut-off value with strong statistical significance could not be identified due to the limited number of cases evaluated. These findings not agree with Rosenwald *et al*. (2001) who found that ZAP-70 expression have been detected in patients with an unmutated status of the Ig variable region genes (IgV_H), who are usually characterized by a more aggressive clinical course and often require therapeutic intervention, Qi *et al*. (2009) who concluded that positive ZAP 70 protein in CLL/SLL suggest a poor prognosis while Carreras *et al*., (2005) found that ZAP-70 positive CLL was associated with a shorter overall survival and a shorter time to disease progression, this discrepancy in overall survival of patients and prognostic significance due to variable protocol therapy, nature of patient, different methodology.

ZAP-70 has raised great interest in CLL patients because it represents a powerful prognostic marker (Durig *et al*., 2003) (Qi *et al*., 2009). Our results provide preliminary indication on the potential use of this protein as a prognostic marker also in ALL. Evaluation of a larger series of patients is needed to further validate this finding.

Finally, the discovery of tyrosine kinesis 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) In Philadelphia chromosome-positive (Ph^+) ALL, the association of imatinib with polychemotherapeutic regimens has resulted in an improved CR rate, disease-free survival and overall survival (Thomas *et al*., 2004). Similarly, the use of epidermal growth factor receptor (EGFR) inhibitors in patients with lung cancer carrying a mutation in the sequence of this receptor has improved the outcome in these patients (Lynch *et al*., 2004).

These results clearly show that ZAP-70 may indeed be a therapeutic target. ZAP-70 expression in a subset of patients with ALL opens the perspective of investigating the use of an inhibitor and can be a candidate molecule for targeted therapy.

CONCLUSION

In conclusion, our results provide evidence that ZAP70 expression in B-lineage ALL increases along the maturation process of B lymphocytes. Furthermore, a high expression of ZAP70 appears to correlate with an increased relapse rate. Finally, these results raise the possibility of designing new compounds targeting this protein.

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