The Egyptian Journal of Hospital Medicine (Jul. 2016) Vol. 64, Page 319- 327

Impact of Circulating Soluble CD40 Concentration Levels in Patients with Hematologic Malignancies

Mohamed Hamed Bahnasawy,Salah el ShahatAref,
Nahed Ahmed Mohamed Omar, Shaimaa Mohamed Ebrahim el Sayed
Zoology Department,Faculty of Science Damietta University ,faculty of Medicine,Mansoura University, Zoology Department,Faculty of Science Damietta University

ABSTRACT
The present study was carried on forty (40) diagnosed ALL (Acute lymphoblastic leukemia), CML (Chronic myeloid leukemia), AML(Acute myeloid leukemia)patients who attended Oncology Centre, Mansoura University. Their ages ranged from 3 to 77 years. They were 27 males and 13 female. Patients were followed up throughout the period of the study. All patients were subjected to the following: Detailed history, clinical examination and Laboratory investigations.

Results: CD40 expression was not associated with any of the studied demographic ،clinical or laboratory variables. No statistically significant associations were elicited between CD 40expression and any of the studied prognostic factors of patients. However ،a significant positive association was detected between patients who responded to chemotherapy and positive CD40L.

Conclusion: CD40L is an independent prognostic factor for relapse free survival ،and also an independent prognostic factor for the prediction of good response to chemotherapy ،since CD40L positive patients are more liable to achieve complete remission ،while CD 40negative ones are more susceptible to death chemotherapeutic resistance.

Key words: CD40, AML,CML,ALL,Hematological Malignancies.

INTRODUCTION

Leukemia are clonal , neoplastic proliferation of immature cells of the hematopoietic system, which are characterized by aberrant or arrested differentiation to increase in the number of blast cells that have common characteristics which induce poor responsiveness to regulatory mechanisms (apoptosis) and tendency to have diminished capacity for normal differentiation and expansion at the expense of normal elements[1].

CD40 is a 50-kDa cell surface protein**,** CD40 is best appreciated as a critical regulator of cellular and humoral immunity via its expression on B lymphocytes, dendritic cells, and monocytes[2].

Aim of the work:

The aim of this work is to assess CD40L in patients with acute lymphoblastic leukemia ، acute myeloid leukemia and chronic myeloid leukemia to evaluate its correlation with the different clinical and laboratory data as well as its relation to disease outcome and prognosis during the period of the study.

MATERIALS AND METHODS

The present study included serum samples from 40 patients, 14 (35%) with ALL, 20 (50%) with AML and 6 (15%) with CML.This cohort comprised 12 males and 28 females with a mean (±SD) age of 34.7 (±22.1) years. In addition, 10 healthy individuals were included as controls.

All patients were subjected to:

a) Detailed history
b) Thorough clinical examination
c) Laboratory investigations:
   1- Complete blood count (CBC).
   2- Bone marrow (BM) aspiration and examination the percentage of BM blast cells.
   3- Evaluate of CD40L by ELISA.

Received: 02/06/2016
Accepted: 12/06/2016
DOI: 10.12816/0029024

Materials supplied in the test kit:

1	Standard(32ng/l)	0.5ml
2	Standard diluent	3ml
3	Microelisa Stripplate	12well×8strips
4	Str- HRP-Conjugate Reagent	6ml
5	30×wash solution	20ml
6	Biotin- sCD40L Ab	1ml
7	Chromogen Solution A	6ml
8	Chromogen Solution B	6ml
9	Stop Solution	6ml
10	Instruction	1
11	Closure plate membrane	2
12	Sealed bags	1

Assay procedure:

1.**Standard dilution:**
this test kit will supply one original Standard reagent, please dilute it by yourself according to the instruction

16ng/mL	Standard No.5	120µl Original Standard + 120µl Standard diluents
8ng/mL	Standard No.4	120µl Standard No.4 + 120µl Standard diluent
4ng/ mL	Standard No.3	120µl Standard No.3 + 120µl Standard diluent
2ng/mL	Standard No.2	120µl Standard No.2 + 120µl Standard diluent
1ng/mL	Standard No.1	120µl Standard No.4 + 120µl Standard diluent

2.The quantity of the plates depends on the quantities of to-be-tested samples and the standards. It is suggested to duplicate each standard and blank well. Every sample shall be made according to your required quantity, and try to use the duplicated well as possible.

Inject sample:
① Blank well: don't add samples and sCD40L -antibody labeled with biotin, Streptavidin-HRP, only Chromogen solution A andB, and stop solution are allowed; other operations are the same.
② Standard wells: add standard 50µl, Streptavidin-HRP 50µl(since the standard already has combined biotin antibody, it is not necessary to add the antibody);
③ To be test wells: add sample 40µl, and then add both sCD40L-antibody 10µl and Streptavidin-HRP 50µl. Then seal the sealing memberance, and gently shaking, incubated 60 minutes at 37 ℃.
Confection: dilute 30 times the 30×washing concentrate with distilled water as standby.
Washing: remove the memberance carefully, and drain the liquid, shake away the remaining water.
Add chromogen solution A 50µl, then chromogen solution B 50µl to each well. Gently mixed, incubate for 10 min at 37℃ away from light.
Stop: Add Stop Solution 50µl into each well to stop the reaction(the blue changes into yellow immediately).
Final measurement: Take blank well as zero , measure the optical densit (OD) under 450 nm wavelength which should be carried out within 15min after adding the stop solution.
According to standards' concentration and the corresponding OD values, calculate out the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample's concentration. It is

acceptable to use kinds of software to make calculation

Statistical analysis

Data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 17.0.

In the statistical comparison between the different groups, the significance of difference was tested using one of the following tests:

1- Man-Whitney test: -Used to compare between two groups of numerical (non-parametric) data.

2- Kruskal Wallis test: -Used to compare between more than two groups of numerical (non-parametric) data.

Spearman's correlation coefficient ® test was used correlating different parameters.

Some investigated parameters were entered into a logistic regression model to determine which of these factors is considered as a significant risk factor and identify its odds ratio.

Univaraite and multivariate survival analyses were performed with the Cox proportional hazards model. Survival curves were constructed according to the Kaplan–Meier method. Finally, a log-rank test was performed to evaluate the statistical significance of differences in survival

RESULTS

The present study included serum samples from 40 patients, 14 (35%) with ALL, 20 (50%) with AML and 6 (15%) with CML as shown in table(3).

This cohort comprised 12 males and 28 females with a mean (±SD) age of 34.7 (±22.1)years. In addition, 10 healthy individuals were included as controls as shown in table(2). Serum levels of CD40L were determined by ELISA. Here, we demonstrated that a proportion of patients with ALL, AML or CML malignancies had significantly (P<0.001) elevated levels (Median =3.70) of the circulating soluble form of CD40Lin comparison with those of controls (Median =1.10). There was no significant difference (P =0.34) in CD40L levels between different types of leukemias as shown in table(1).

Using 3.7 ng/ml as cutoff level, the prognostic value of CD40L was evaluated by comparing the overall survival of the $CD40L^{Low}$ group (<3.7ng/ml) with that of the $CD40L^{High}$ group (>3.7ng/ml). Patients in the$CD40L^{High}$ group had a significantly shorter survival (P =0.009) than patients in the $CD40L^{Low}$group.In univariate analysis, other variables that were associated significantly with survival were blast cell count (P =0.034) and remission (P =0.003). The independence of the prognostic value of CD40L levels was evaluated by using the multivariate Cox regression model. Among all tested prognostic factors, only the level of CD40L ($CD40L^{High}$vs. $CD40L^{Low}$) was a significant independent prognostic factor in this model (P<0.033) with high hazard ratio (4.224; 95% CI (1.121-15.923) as shown in table(15).

Elevated levels were associated with significantly shorter treatment free and overall survival as shown in tables(13,14).

There is no significant difference in CD40L serum levels between patients with normal liver and those with enlarged liver (P =0.22). Similarly, there is no significant difference in CD40L levels between patients with normal, large or removed spleen (P =0.25)as shown in table (5). CD40L serum levels significantly (P<0.0001) correlated (r =0.606) with blast cell count as shown in table(15).

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The trans-membrane molecule CD40 has attracted attention as a therapeutic target in leukemia malignancies. Within the haematopoietic system, the CD40-CD40L interaction plays a central role in immune regulation[3].

Studies on CD40L have revealed that it enhances antineoplastic immune response of the body, inhibits tumor growth, and induces apoptosis of cancer cells. The effect induced by CD40L has appeared to depend not only

on the type of cells that show the receptor expression but also on the strength of the signal transmitted by the ligand. High signal (the cell has many CD40 molecules) indicates apoptosis of cancer cells, whereas low signal (a small number of receptors) CD40L promotes cancer growth [4].

Fas ligand and CD40L are transmembrane proteins that are expressed predominantly on activated T lymphocytes. The malignantchronic lymphocytic leukemiacells express CD40 and Fas receptors, which can transduce cell-survival and cell-death signals. Thus, **Younes*et al*.**[5]examined the role of CD40 in the growth regulation of chronic leukemiacells and its interaction with Fas-mediated and fludarabine-induced apoptosis *in vitro*.

Chronic leukemiacells underwent apoptosis in culturewhich was enhanced by Fas ligand.While, CD40L rescued chronic leukemiacells from spontaneous apoptosis and caused malignant cells to resist apoptosis induced by FasL. The mean soluble CD40L level was significantly elevated (P<0.001)in chronic leukemiapatients compared to the normal donors. These results demonstrated that serum of patients with chronic lymphocytic leukemiacontained elevated levels of biologically active soluble CD40L [5]. Under most circumstances, chronic leukemiaB cells do not proliferate in culture and express a limited repertoire of surface antigens, including CD19, CD20, CD23, CD27, CD40, and CD70. While, **Schattner et al.**[6]reported that freshly isolated B cells from a subset of chronic leukemiacases constitutively express CD40L, a member of the tumor necrosis factor family which is normally expressed by activated CD4(+) T cells and mediates T-cell-dependent B-cell proliferation and antibody production.

CD40L was detected in purified chronic leukemiaB cells by immunofluorescence flow cytometry, by RT-PCR, and by immunoprecipitation. To demonstrate that CD40L in the CLL B cells is functional, they used irradiated chronic leukemiacells to stimulate IgG production by target, nonmalignant B cells in coculture. The chronic leukemiaB cells induced IgG production by normal B cells to a similar degree as did purified T cells in a process which was partially inhibited by monoclonal antibody to CD40L[6].

Using 3.7 ng/ml as cutoff level, the prognostic value of CD40L was evaluated by comparing the overall survival of the $CD40L^{Low}$ group (<3.7ng/ml) with that of the $CD40L^{High}$ group (>3.7ng/ml). Patients in the$CD40L^{High}$ group had a significantly shorter survival (P =0.009) than patients in the $CD40L^{Low}$group.In univariate analysis, other variables that were associated significantly with survival were blast cell count (P =0.034) and remission (P =0.003). The independence of the prognostic value of CD40L levels was evaluated by using the multivariate Cox regression model. Among all tested prognostic factors, only the level of CD40L ($CD40L^{High}$vs. $CD40L^{Low}$) was asignificant independent prognostic factor in this model (P<0.033) with high hazard ratio (4.224; 95% CI (1.121-15.923)).

Themajority of chronic leukemia cells strongly express membrane CD40 (mCD40), and mCD40-CD40L engagement within the lymph node microenvironment is thought to provide signals critical for their proliferation and survival[3] .It has become evident that high levels of proliferation of the leukemic population in chronic leukemiaare correlated with worse prognosis. In proliferation centers, chronic leukemiacells are in close contact with activated CD40L+ CD4+ T cells, and it has been proposed that these cells can support the growth of chronic leukemiacells through CD40 ligation.However, although CD40L stimulation alters the apoptotic profile of chronic leukemiacells and increases their resistance to apoptosis, it induces minimal proliferation on its own.Thus, there might be other stimuli provided by activated CD4+ T cells that contribute to proliferation of chronic leukemiacells[7].

So, increased levels of CD40L may cause in shorter survival.

Hock et al.[8]reported thatmany patients with hematologic malignancies have elevated circulating levels of soluble CD40, and these elevated levels are associated with a poor prognosis especially in patients with multiple myelomaand acute myeloid leukemia, suggesting that CD40 may have a role in modulating antitumor responses and also may

be a useful prognostic marker.Moreover, **Hock *et al*.**[3] investigated the prognostic significance of plasma CD40 in untreated chronic leukemiapatients. They reported that most of patients had levels higher than those of normal donors and that elevated levels were associated with significantly shorter treatment free and overall survival. These results suggestedthat CD40 may play a role in chronic leukemiaprogression.

Serum CD40L levels not correlate with some established prognostic factors like age, hemoglobin or platelet counts. Similar findings were obtained by **Lee *et al*.**[9]who reported that plasma CD40L levels not correlated with these parameters in sickle cell anemia. Else, theprognostic value of CD40L levels in ALL, AML and CML appears to be independent of other established prognostic factors like spleen and liver size.

There is no significant difference in CD40L serum levels between patients with normal liver and those with enlarged liver (P =0.22). Similarly, there is no significant difference in CD40L levels between patients with normal, large or removed spleen (P =0.25). These results suggest that, irrespective of its actual functional roles, CD40L merits further investigation as a clinically useful prognostic marker not only in ALL, AML and CML but also in chronic lymphatic leukemia[8].
Moreover, CD40L serum levels significantly (P<0.0001) correlated (r =0.606) with blast cell count. Else, CD40L levels significantly correlated with WBC count as another marker of inflammation. Similar correlation between CD40L and WBC was obtained by **Unek*et al*.**[10].

CONCLUSION

The data presented in the current study demonstrate that significantly elevated levels of a circulating, soluble form of CD40L are present in patients with ALL, AML and CML malignancies and are associated with poor prognosisandshorter survival.
Recommendation,these results further suggest that CD40Lis a potential prognostic biomarker for different types of leukemiamalignancies. Thus, we can recommend the detection of soluble CD40Las individual or in a combination with other established prognostic markers tonot only provides useful information for therapeutic supervision judgment but also to monitoring the disease.

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Table (1): association between Median and IQR in cases and control.

		Groups		P
		Control	Cases	
CD40L	Median	1.10	3.70	<0.001
	IQR	.90-2.50	2.90-4.65	

P: Probability Test used: Mann-Whitney U test IQR: Interquartile range

There is high significant difference between leukemia cases and controlCases.

Table (2): description of Mean and SD regarding age and sex.

Age	Mean		34.67
	±SD		22.13
sex	Male	No	12
		%	30.0%
	Female	No	28
		%	70.0%

SD: Standard deviation

Table (3): Percentage of diagnosed types of leukemia.

		No	%
diagnosis	ALL	14	35.0%
	AML	20	50.0%
	CML	6	15.0%

Table (4): Descriptions of CD40L (Hb, WBCs, Plts, Blast cell count«BM, Peripheral» and LDH) represented as Median and Range.

	Median	range
Hb(g/dl)	9.10	2.30-15.10
WBCs	5.65	.03-230.00
Platelets	22.00	.15-370.00
Blast cell count BM%	42.00	.00-100.00
BLAST CELL COUNT P%	10.00	.00-95.00
LDH	699.08	1.00-3600.00

Table (5): Frequency of Clinical findings (Liver, Spleen and LN) in studied cases.

		No
Liver	Normal	13
	Enlarged	27
SPLEEN	Normal	12
	Enlarged	26
	Removed	2
LN	N	29
	P	11

Table (6): Frequency of Remission induction, Outcome die and Time of the study (Mean, ±SD).

remission induction	**No**	**No**	**16**
		%	**40.0%**
	Yes	**No**	**24**
		%	**60.0%**
outcome died	**Live**	**No**	**16**
		%	**40.0%**
	Die	**No**	**24**
		%	**60.0%**
Time	**Mean**		**14.90**
	±SD		**8.58**

CD40 vs others:

Table (7): Comparison between Male and Female in CD40L expression (Median-IQR).

		sex		P
		Male	**Female**	
CD40L	**Median**	**3.40**	**3.95**	**0.38**
	IQR	**2.80-4.00**	**3.00-4.90**	

Test used: Mann-Whiney test

Table (8) Comparison between ALL, AML and CML in CD40L expression (Median-IQR).

		ALL	AML	CML	P
CD40L	**Median**	**3.80**	**3.25**	**4.00**	**0.34**
	IQR	**3.50-4.80**	**2.70-4.75**	**3.50-5.25**	

Test used: Kruskalwallis test

Table (9): Comparison between Normal and Enlarged Liver in CD40L expression (Median and IQR).

		Liver		P
		Normal	**Enlarged**	
CD40L	**Median**	**3.45**	**4.10**	**0.22**
	IQR	**2.80-4.00**	**2.90-5.90**	

Test used: Mann-Whiney test

Table (10) Comparison between Normal, Enlarged and Removed Spleen in CD40L expression (Median and IQR).

		SPLEEN			P
		Normal	**Enlarged**	**Removed**	
CD40L	**Median**	**4.00**	**3.50**	**4.10**	**0.25**
	IQR	**3.40-5.60**	**2.70-4.40**	**3.70-4.50**	

Test used: Kruskalwallis test

Table (11): Comparison between cases with Free LN and those with Positive symptoms and signs in CD40L expression (Median and IQR).

		LN		P
		N	P	
CD40L	Median	3.70	4.10	1.00
	IQR	2.90-4.80	2.90-4.40	

Test used: Mann-Whiney test

Table (12): Comparison between cases with remission and those who are relapsing in CD40L expression (Median and IQR).

		Remission Induction		P
		No	Yes	
CD40L	Median	4.35	3.10	<0.001
	IQR	3.95-6.15	2.70-3.70	

Test used: Mann-Whiney test

There is significance difference in CD40L levels between cases with remission and those who are relapsing.

Table (13): Comparison between Live and Dead (outcome died) in CD40L expression (Median and IQR).

		outcome died		P
		Live	Die	
CD40L	Median	2.80	4.30	<0.001
	IQR	2.70-3.30	3.75-6.15	

Test used: Mann-Whiney test
There is significance difference in CD40L levels between live and Dead cases.

Logistic regression(Stepwise):

Table (14): CD40L Logistic regression.

	P	OR	95% C.I.
CD40L	.009	4.108	1.421-11.879

P: Probability OR: Odds ratio CI: Confidence interval

The serum level of CD40L was a significant independent prognostic factor.

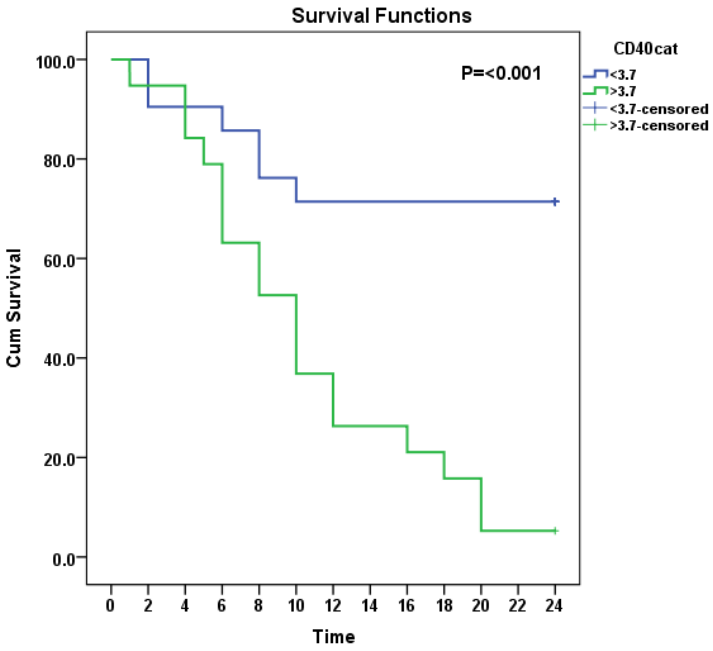

Figure (1): Kaplan Meier curve for patients with positive CD40.

Table (15):Cox regression (Univariate Analysis,Multivariate Analysis)

		Univariate			Multivariate		
		P	HR	95% C.I.	P	HR	95% C.I.
Age		0.75	1.003	0.98-1.025			
Sex	Female	.089	2.357	.877 - 6.337			
	Male						
HB		.995	1.000	.878 - 1.138			
WBC		.952	1.000	.992 - 1.007			
Platelets		.70	1.001	.997 -1.005			
Blast cell count BM		.034	1.017	1.001-.033	.508	1.006	.989-1.023
BLAST CELL COUNT P		.381	1.006	.993- 1.018			
LDH		.667	1.000	.999-1.000			
HEPATO		.281	1.720	.642-4.613			
SPLEEN		.773	.893	.413-1.929			
LN		.190	1.814	.745-4.416			
Remission		.003	.265	.112-.628	.465	.670	.229-1.959
CD40	>3.7	<0.001	6.245	2.273- 17.158	.033	4.224	1.121-15.923
	<3.7						

P: Probability HR: Hazard ratio CI: confidence interval

***Table (16):*Correlation between CD40 and age,HB,WB,Platlets,Blast cellcount BM%,BLAST CELL COUNT P%,LDH,Time**

		age	Hb (g/dl)	WB	platlets	Blast cellcount BM%	BLAST CELL COUNT P%	LDH	Time
CD40L	r	-.107	.040	-.366-*	-.254	.606**	.194	.140	.190
	P	.516	.805	.020	.113	<0.001	.231	.390	.374

r:Spearman correlation coefficient P:probability