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Immunohistochemical Expression of CD27 in Bone Marrow Biopsies of Chronic Lymphocytic Leukemia Patients

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Abstract:

Background:

Chronic lymphocytic leukemia (CLL) is the most frequent lymphoproliferative disorder in western world. Its main characteristic is proliferation of mature looking lymphocytes in bone marrow, blood and lymphoid tissues. Diverse clinical course, ranging from indolent course to aggressive disease could be seen .So, many efforts focus on finding reliable indicators that can help to predict the outcome or explain CLL clinical variability.

Certain surface proteins such as CD38, CD49d and ZAP-70 are expressed on leukemic cell population and they are widely used as well-established prognosticators. Novel Markers such as CD27 has been emerged, but still there is controversy about its exact role in CLL prognosis.

CD27 molecule is a member of the TNF receptor family, related to leukemic cells adherence to stromal cells, and to CLL prognostic factors.

This study aimed to assess the immunohistochemichal expressions of CD27 in bone marrow biopsies of CLL patients and correlate it to the clinicopathological parameters.

Methods:

Cross sectional study was done on forty bone marrow paraffin blocks of untreated patients with CLL, collected from teaching laboratories of Medical City and National Center of Hematology, from November 2016 to June 2018. Immunohistochemical staining for CD27, CD38 antibodies were studied in relation to clinicopathological details.

Results:

Positive result of CD27 was found in (77.5%) of cases, with moderate intensity in (87.1%), score (1) in (61.3%) of cases. CD27 was significantly associated with Binet stage C, Rai stage IV , Hb level less than 10 g/dl and diffuse bone marrow involvement with (P =0.021, 0.001, 0.018, and 0.001 respectively). No association could be established between CD27 and the well-established CD38 in our study group.

The sensitivity of CD27 was (100%) for CLL high risk patients (Binet stage C). Specificity = 33.3% and accuracy of CD27 marker was 55%.

Conclusions and recommendations:

Immunohistochemistry can be used reliably to assess the expression of CD markers. Positive expression of CD27 in CLL cases significantly correlated with more advanced stage and CLL histological type of poor prognosis.

We recommend further prospective studies to address if there is possible correlation between CD27 in CLL and chemosensitivity and other prognostic indicators.

Keyword: *Chronic lymphocytic leukemia, CLL prognosis, CD27, CD38, CLL immunohistochemistry.*

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Introduction:

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia of adults in western countries. The hall mark of CLL is the proliferation of mature looking and immunologically defective B lymphocytes in bone marrow, blood and lymphoid tissues. Clinically it has extremely hetero-geneous course, with some patients deteriorate quickly with aggressive disease, while others with an indolent course and do not require therapy (1). So, all the efforts focus on finding reliable indicators that can help to predict the outcome or explain CLL clinical variability. Those prognosticators include expression of several proteins in the leukemic lymphocytes, immunoglobulin heavy chain variable region gene (IGHV) mutational status, cytogenetics and the response to treatment (2)we sequenced this gene in 565 patients. NOTCH1 mutations, found in 63 patients (11%.

Expression of surface proteins such as CD38, CD49d and ZAP-70 on leukemic cells has been proven as a dependable prognosticator in many studies. At the moment, CD38 and ZAP-70 are widely used in the prog-nostic plan of CLL patients (3). Apart from the well-established markers (mentioned above), many new cell markers such as CD27 and others have been emerged, but they need thorough assessment for their prognostic capacity in CLL (4).

CD27 molecule is a member of the TNF receptor family. It is specifically expressed on cells of lymphoid origin, including naive CD4+ and CD8+ T cells; yet it is also expressed on B cells, T cells, NK cells and dendritic cells (DCs), but only upon stimulation(5). The main effect of CD27 molecule is to provide a co-stimulatory signal for T and B cells activation after cross-linkage with CD70 (6). The multi-task effect of CD27on immune cells suggests the possibility of its role in cancers. CD27 signaling has been linked to tumor pathogenesis in chronic myeloid leukemia (CML) (7), non-Hodgkin B cell lymphoma and chronic lymphocytic leukemia (CLL) (8)we analyzed the role of the CD27-CD70 interaction in the immunologic control of solid tumors in Cd27-deficient mice. In tumor-bearing wild-type mice, the CD27-CD70 interaction increased the frequency of regulatory T cells (Tregs.

Although CLL patients express membranous as well as soluble forms of CD27, little is known about the role of this marker in CLL regulation and progression. Recent studies estimated soluble CD27 level and find a concordance between elevated soluble CD27 in CLL patients and functional capacity of leukemic cells to adhere to stromal cells, and other prognostic factors such as serum β2-microglobulin; thus proposing its prognostic value. Membranous form of CD27 and its impact on CLL is an interesting issue to address (9,10)in concert with T-cell receptor crosslink-ing, can induce T-cell proliferation and cellular immune acti-vation. We find that chronic lymphocytic leukemia (CLL.

This study aimed to assess membrane expression of CD27 in bone marrow biopsies of CLL patients and investigate its correlation with other clinic-path ological parameters.

Subject and Method:

Cross sectional study was intended, which include 40 bone marrow paraffin blocks from newly diagnosed CLL patients. These blocks were retrospectively collected from teaching laboratories of Medical City and National Center of Hematology in Baghdad. These biopsies were related to the period from November 2016 to June 2018.

Related clinico-pathological details including age and sex of patients, Hb , absolute lymphocyte count, platelet count , percentage of lymphocyte in the bone marrow and histological type were obtained by the researcher from patients' admission case sheets and laboratory pathology reports that were kept in hospitals archives. This was done after having a written permission to access all these data and specimens.

Immunohistochemistry was used to detect and localize CD27 in CLL BM tissue sections. From each block, three sections were taken, one stained with hematoxylin and eosin (H & E) and the other two were stained immunohistochemically for CD27 and CD38.

IHC technique was carried out using rabbit monoclonal anti-CD27 antibody [(ab131254), Abcam], anti-CD38 antibody [(ab108403), Abcam] and secondary detection kit [Rabbit specific HRP/DAB (ABC) (ab64261), Abcam]. IHC protocol was done according to manufacturer's instructions. Positive control used for CD27 was gastric tissue figure (1), and tonsiller tissue used as positive control for CD38 figure (2), while negative control was obtained by omitting the primary antibody and replaced by phosphate- buffer saline (PBS) figure (3).

Positive results were indicated by distinct brown membranous precipitate of leukemic lymphocytes (11). The immunohistochemical expression of CD27 and CD38 positivity was analyzed in a semi-quantitative scheme. This system depends on two variables, percentage of stained lymphocytes (0: negative, 1: ≤10%, 2: 11-50%, 3: >50%) and intensity of staining (0: negative, 1: mild, 2: moderate, 3: strong) (12) (13).

Statistical analysis:

The data were analyzed using Statistical Package for Social Sciences (SPSS) version 25. The data presented as mean, standard deviation and ranges. Categorical data presented by frequencies and percentages. Independent t-test and two tailed was used to compare the continuous variables among study groups accordingly. Pearson's Chi–square test was used to assess statistical association between different variables. A level of P – value less than 0.05 was considered significant.

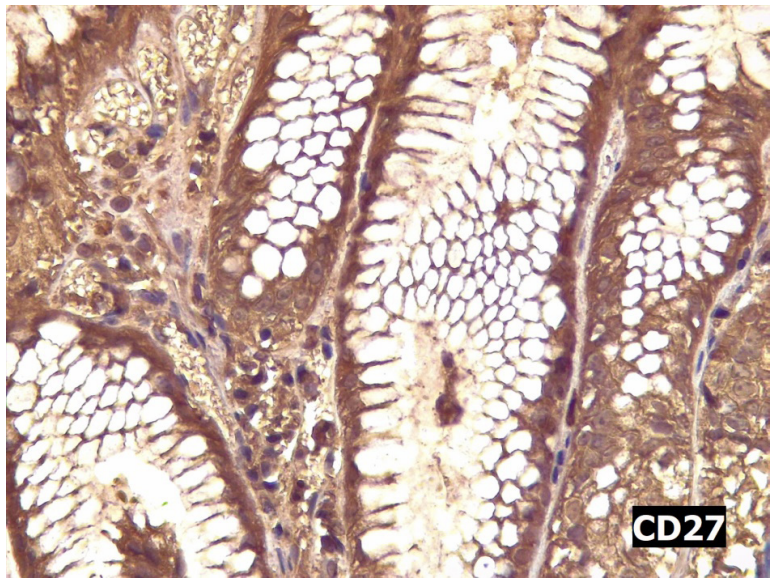

Figure 1: IHC expression of CD27 in gastric tissue section as a positive control. Diffuse brown cytoplasmic and membranous expression of CD27 monoclonal antibody in gastric glands epithelial cells (arrows) (40X).

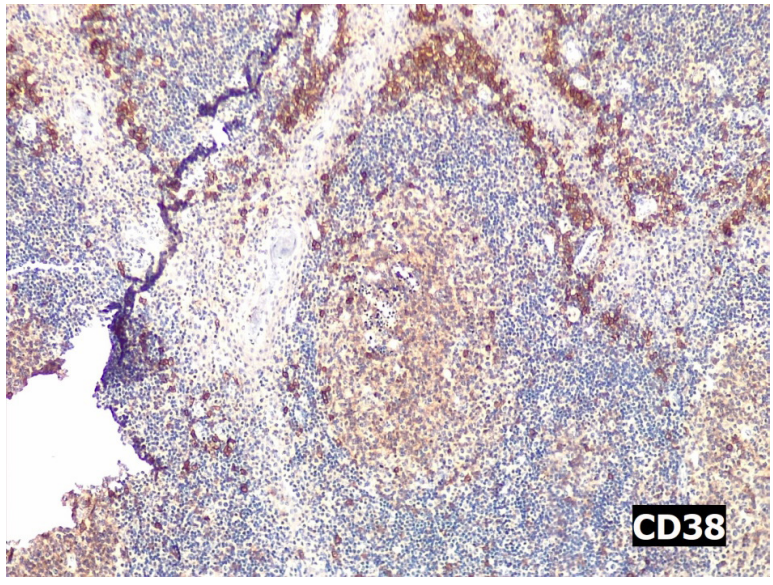

Figure2: IHC expression of CD38 in normal tonsiller tissue section as a positive control. Diffuse brown cytoplasmic and/or membranous expression of CD38 monoclonal antibody in B lymphocytes (arrows) (10X).

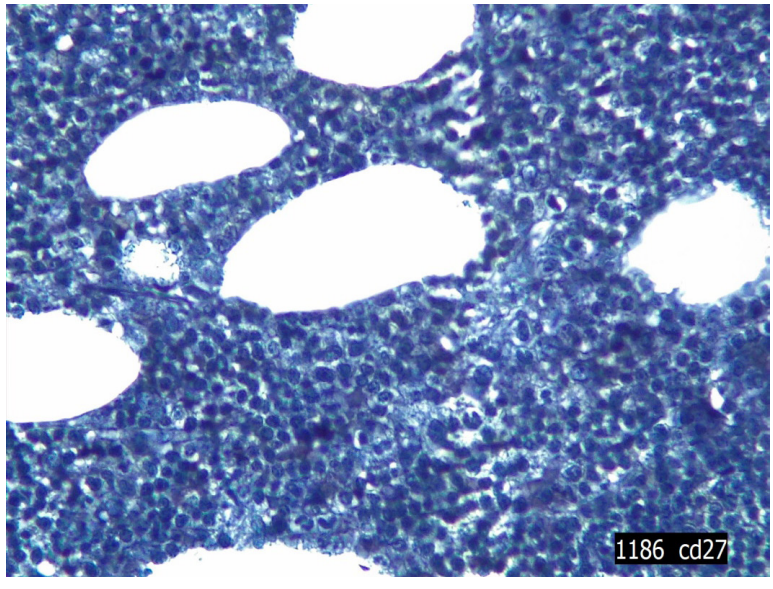

Figure3: Technical negative control for CD27 IHC marker in BM of CLL. It shows negative expression of marker (arrows) (40X).

Results:

Study patients were 27 males and 13 females with their age ranging from 28 to 75 years (mean of 57.18 years ± 11.27 years). The highest proportion of study patients was found in the age group > 60 years (52.5%) table 1.

Table 1: Distribution of study patients by general characteristics

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Variable	No. (n=40)	Percentage (%)
Age (Years)		
< 40	2	5
40 – 60	17	42.5
> 60	21	52.5
Gender		
Male	27	67.5
Female	13	32.5

Hemoglobin level was less than 10 g/dl in about one thirds of study patients (32.5%). Most of patients (82.5%) were with platelets count more than 100* 109/l.

Absolute lymphocyte count range from 6.9x 109- 255x 109/l. Patients distribution according to Binet and Rai staging systems are shown in figures4and5.

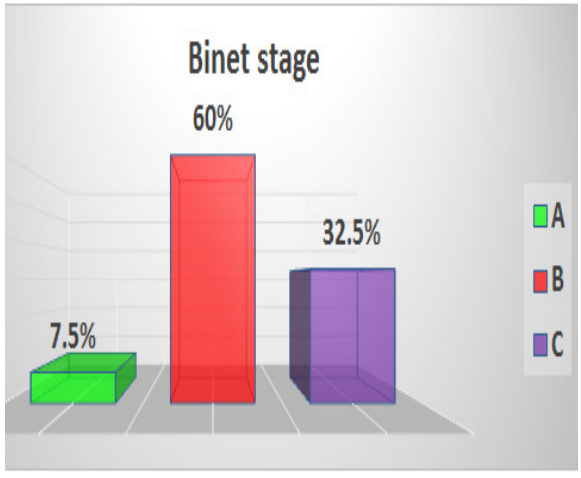

Figure 4: Distribution of study patients according to Binet staging system

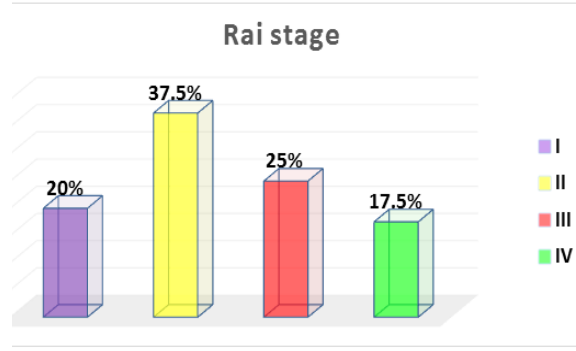

Figure 5: Distribution of study patients by Rai staging system

CD27 expression and its correlation with prognostic signs:

The distribution of study patients according to CD27 marker details is shown in table 2. Positive result of this marker was found in more than three quarters of study patients (77.5%). Moderate intensity was the most common (87.1%), while the score (1) was the most prevalent (61.3%).

CD27 expression and its correlation with prognostic signs:

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Table 2: Distribution of study patients by CD27 marker details

CD 27 Marker Details	No. (n=40)	Percentage (%)
Results		
Positive	31	77.5
Negative	9	22.5
(Details of positive cases No. (n=31		
Intensity	No. (n=31)	
Weak	3	9.7
Moderate	27	87.1
Strong	1	3.2
Scoring		
Score 1	19	61.3
Score 2	9	29.0
Score 3	3	9.7

The association between CD27 marker intensity and bad prognostic signs is shown in table 3. All patients with Rai stage IV revealed moderate intensity of CD27 marker (100%), while moderate intensity represented the highest proportion (88%) among patients with diffuse type of bone marrow involvement with a significant association between each of bone marrow involvement and Ria stage and prevalence of CD27 marker intensity (P= 0.001).

It was clear that there was no significant association (P ≥ 0.05) between CD27 marker intensity and each of Binet stage, Hb level, and platelets count. CD27 staining intensities are shown in the figures 6,7 and 8.

Table 3: Association between prevalence of CD27 marker intensity and prognostic signs

Bad Prognostic Signs	CD27 Marker Intensity				(%) Total n= 40	P- Value
	(%) Negative n= 9	(%) Weak n= 3	(%) Moderate n= 27	(%) Strong n= 1		
Binet Stage						
A	(66.7) 2	(0) 0	(33.3) 1	(0) 0	(7.5) 3	**0.178**
B	(29.2) 7	(8.3) 2	(58.3) 14	(4.2) 1	(60.0) 24	
C	(0) 0	(7.7) 1	(92.3) 12	(0) 0	(32.5) 13	
Rai Stage						
I	(75.0) 6	(12.5) 1	(12.5) 1	(0) 0	(20.0) 8	**0.007**
II	(20.0) 3	(6.7) 1	(73.3) 11	(0) 0	(37.5) 15	
III	(0) 0	(10.0) 1	(80.0) 8	(10.0) 1	(25.0) 10	
IV	(0) 0	(0) 0	(100.0) 7	(0) 0	(17.5) 7	

Bone Marrow Involvement						
Diffuse	(0) 0	(8.0) 2	(88.0) 22	(4.0) 1	(62.5) 25	**0.001**
Interstitial	(50.0) 6	(8.3) 1	(41.7) 5	(0) 0	(30.0) 12	
Nodular	(100.0) 3	(0) 0	(0) 0	(0) 0	(7.5) 3	
Hb Level g/dl						
10 >	(0) 0	(7.7) 1	(92.3) 12	(0) 0	(32.5) 13	**0.087**
10 ≤	(33.3) 9	(7.4) 2	(55.6) 15	(3.7) 1	(67.5) 27	
Platelets Count/l						
10^9*100 >	(0) 0	(0) 0	(100.0) 7	(0) 0	(17.5) 7	**0.252**
10^9*100 ≤	(27.3) 9	(9.1) 3	(60.6) 20	(3.0) 1	(82.5) 33	

The association between CD27 marker scoring and bad prognostic signs is shown in table 4. We found that, score (1) represented the highest prevalence in patient with Binet stage B (58.3%), Rai stage II (66.7%), diffuse type of bone marrow involvement (52%), and Hb level more than 10 g/dl (55.6%) with a statistically significant association (P=0.005, 0.003, 0.001, and 0.001 respectively) between these four bad prognostic signs and prevalence of CD27 marker scoring. There was no significant association (P = 0.258) between CD27 marker scoring and platelets count.

Table 4: Association between prevalence of CD27 marker scoring and prognostic signs

Prognostic Signs	CD27 Marker Scoring				(%) Total n= 40	P- Value
	(%) Negative n= 9	(%) Score 1 n= 19	(%) Score 2 n= 9	(%) Score 3 n= 3		
Binet Stage						
A	(75.0) 2	(25.0) 1	(0) 0	(0) 0	(7.5) 3	**0.005**
B	(29.2) 7	(58.3) 14	(12.5) 3	(0) 0	(60.0) 24	
C	(0) 0	(30.8) 4	(46.1) 6	(23.1) 3	(32.5) 13	
Rai Stage						
I	(75.0) 6	(25.0) 2	(0) 0	(0) 0	(20.0) 8	**0.003**
II	(20.0) 3	(66.7) 10	(13.3) 2	(0) 0	(37.5) 15	
III	(0) 0	(40.0) 4	(40.0) 4	(20.0) 2	(25.0) 10	
IV	(0) 0	(42.9) 3	(42.9) 3	(14.2) 1	(17.5) 7	
Bone Marrow Involvement						
Diffuse	(0) 0	(52.0) 13	(30.0) 9	(12.0) 3	(62.5) 25	**0.001**
Interstitial	(50.0) 6	(50.0) 6	(0) 0	(0) 0	(30.0) 12	
Nodular	(100.0) 3	(0) 0	(0) 0	(0) 0	(7.5) 3	
Hb Level g/dl						
10 >	(0) 0	(30.8) 4	(46.1) 6	(23.1) 3	(32.5) 13	**0.001**
10 ≤	(33.3) 9	(55.6) 15	(11.1) 3	(0) 0	(67.5) 27	
Platelets Count/l						
10^9*100 >	(0) 0	(42.9) 3	(24.9) 3	(14.2) 1	(17.5) 7	**0.258**
10^9*100 ≤	(27.3) 9	(48.5) 16	(18.2) 6	(6.0) 2	(82.5) 33	

The association between CD27 marker results and bad prognostic signs is shown in table 5. All patients with Binet stage C, Rai stage III and IV, bone marrow involvement (diffuse type), and Hb level less than 10 g/dl showed positive CD27 marker result with a significant association (P= 0.021, 0.001, 0.001 and 0.018 respectively) between these four bad prognostic signs and prevalence of CD27 marker result.

We found that there was no significant association (P=0.117) between CD27 marker result and platelets count.

Table 5: Association between prevalence of CD27 marker results and bad prognostic signs

Bad Prognostic Signs	CD27 Marker Results		(%) Total n= 40	P- Value
	(%) Positive n= 31	**(%) Negative n= 9**		
Binet Stage				
A	(33.3) 1	(66.7) 2	(7.5) 3	**0.021**
B	(70.8) 17	(29.2) 7	(60.0) 24	
C	(100.0) 13	(0) 0	(32.5) 13	
Rai Stage				
I	(25.0) 2	(75.0) 6	(20.0) 8	**0.001**
II	(80.0) 12	(20.0) 3	(37.5) 15	
III	(100.0) 10	(0) 0	(25.0) 10	
IV	(100.0) 7	(0) 0	(17.5) 7	
Bone Marrow Involvement				
Diffuse	(100.0) 25	(0) 0	(62.5) 25	**0.001**
Interstitial	(50.0) 6	(50.0) 6	(30.0) 12	
Nodular	(0) 0	(100.0) 3	(7.5) 3	
Hb Level g/dl				
10 >	(100.0) 13	(0) 0	(32.5) 13	**0.018**
10 ≤	(66.7) 18	(33.3) 9	(67.5) 27	
Platelets Count/l				
10^9*100 >	(100.0) 7	(0) 0	(17.5) 7	**0.117**
10^9*100 ≤	(72.7) 24	(27.3) 9	(82.5) 33	

In this study, the mean of absolute Lymphocytes Count was significantly higher in patients with score (3) of CD27 marker than that in patients with each of score 1 and 2 (171.76 versus 75.54 and 31.87, P=0.001) table 6.

Table 6: Relation between CD27 marker expression and absolute lymphocyte count

Variable	Absolute Lymphocytes Count .Mean ± Std. Dev	P-Value
CD27 Marker Intensity		
Weak	63.99 ± 54.98	**0.960**
Moderate	67.14 ± 59.08	
Strong	40.60	
CD27 Marker Scoring		
Score 1	28.02 ± 31.87	**0.001**
Score 2	46.93 ± 75.54	
Score 3	140.42 ± 171.76	
CD27 Marker Results		
Positive	64.74 ± 58.09	**0.317**
Negative	44.70 ± 34.62	

Relation between CD27 and CD38 expression:

Table 7 shows the association between CD27 marker result and CD38 marker result. No significant association (P=0.394) between CD27 marker and CD38 marker results.

Table 7: Association between CD27 and CD38 markers results

CD27 Marker Result	CD38 Marker Results		Total (%) n= 40	P- Value
	Positive (%) n= 31	**Negative (%) n= 9**		
Positive	(80.6) 25	(19.4) 6	(77.5) 31	**0.394**
Negative	(66.7) 6	(33.3) 3	(22.5) 9	

Sensitivity, specificity, and accuracy of CD27 Marker according to Binet stage:

The sensitivity = 100%, specificity = 33.3% and accuracy of CD27 marker was 55% as shown in table 8.

Table 8: Sensitivity, specificity, and accuracy of CD27 marker

CD27 Marker Result	**Binet Stage**		**Total**
	High Risk	**Low Risk**	
Positive	13	18	31
Negative	0	9	9
Total	13	27	40

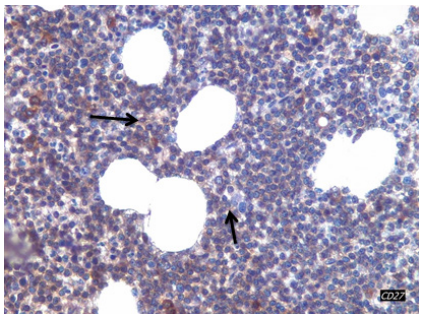

Figure 6: IHC expression of CD27 in BM tissue section of CLL (mild intensity). Leukemic lymphocytes diffusely show cytoplasmic and membranous CD26 light brown immunostain (arrows) (40X).

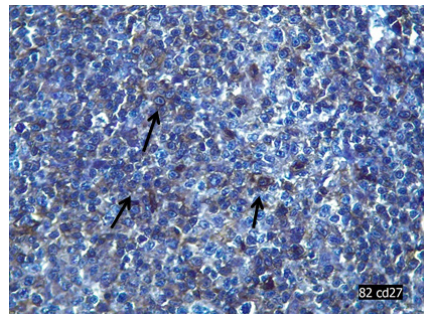

Figure 7: IHC expression of CD27 in BM tissue section of CLL (moderate intensity). Leukemic lymphocytes diffusely show cytoplasmic and membranous CD27 immunostain (arrows) (40X).

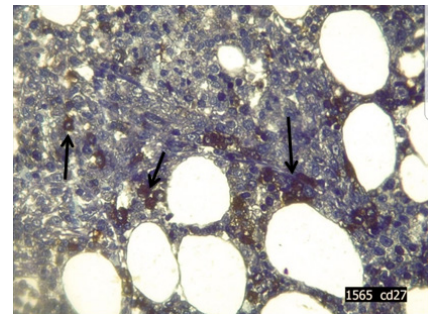

Figure 8: IHC expression of CD27 in BM tissue section of CLL (strong intensity). Positive lymphocytes display dark brown-black cytoplasmic and membranous CD27 immunostain in an interstitial pattern (arrows) (40X).

Discussion:

This study included 40 CLL patients with a mean age of 57.18 ± 11.27 years, 52.5% of them were above the age of 60 years. This was close to Iraqi study performed by Naji (15).

The percentage of patients between the ages of 40 to 60 years was 42.5%, which was higher than what reported in other studies from Europe and USA in which only 5%–11% of CLL cases at diagnosis were younger than 55 years (16–19).

These differences could be attributed to different racial groups, different sample size, and inclusion or exclusion criteria in their studies as well as environmental factors.

The male: female ratio was about 2:1, and this was consistent with the nature of disease and what published in other studies (20–22). However, Naji stated higher male to female ratio 3.5:1 (15).

Upon staging, 37.5% of our study patients were Rai stage II and 42.5% were stage III and IV. This was parallel with Naji results in Iraq (15). In Binet staging system the majority were stage B, C and only 7.5% were stage A, and this is opposite to the situation in France, Brazil and United States in which majority of cases were in the early stages (23–25).

The fact that majority of our study patients were in late stages of CLL, unlike other international studies, could be explained in many ways. First, late detection of our patients because of ignorance of early vague symptoms and poor health services. Second, about half of our sample were younger than 60 years (40-60years). And it was concluded that CLL in younger ages specially those ≤55 years old (median, 50 years) tends to show more aggressive course (17).

IHC expression of CD27 and correlation with prognostic signs:

CD27 is a surface protein related to tumor necrosis factor (TNF) receptor family that is known to be expressed on germinal center B lymphocytes after facing antigen. CD27 signaling via CD70 is important for B cell co-stimulation. This pathway is tightly regulated, If this control is disrupted ,then CD27-CD70 interaction will support tumor progression. CD27 has been reported to be expressed by certain hematological malignancies such as CLL and was linked to poor outcome (26).

The results of the present study revealed that CD27 score significantly correlates with higher stages of CLL according to both Binet and Rai's clinical staging system with p values 0.021 and 0.001 respectively. Also, there is a statistically significant relation between CD27 and histological type of poor prognosis. All patients with nodular BM involvement (3 cases) were negative for CD27.On the other hand, all cases with diffuse pattern (25 cases) showed positive expression. While those with interstitial BM involvement (12 cases) displayed heterogenous expression. Furthermore, our outcome demonstrated a positive correlation with lower Hb, and no correlation with platelets table 5.

According to what mentioned above, it is clear that CD27 expression is associated with poor prognostic signs. This agreed with previously published studies. Abouzeid et al. who assessed soluble CD27 in CLL patients by ELISA, found higher level of sCD27 in leukemic patients in comparison with control group. In addition, patients who had higher serum levels of sCD27 failed to achieve complete response CR to therapy in contrast to those with lower sCD27 levels, so it can predict response to treatment as well (27).

The current study is consistent with Lafarge et al. who stated that CLL patients with ZAP-70 expression (which is a marker of bad prognosis) showed significantly higher expression of CD27 than did ZAP-70 negative group. And he explained the concordant relation between CD27 reactivity and the capability of CLL to adhere to stromal cells and the blockade of CD27 prevent binding of leukemic cells to stroma. This fact propose CD27 as an important potential target for immunomodulatory drugs (10).

Turkish study performed by Kara et al (2007) also mentioned that sCD27 expression (ELISA) was higher in CLL patients in comparison with healthy donors. The same researchers found that sCD27 levels were significantly correlated with Rai's clinical staging system (p=0.008), HB levels (p=0.028), B2M (P=.000) and lactate dehydrogenase (P=.001).(28) These findings were parallel ours in that CD27 expression positively correlates with aggressive clinical course.

Another finding which has been addressed in our study is that CD27 immunohistochemical score correlate with higher lymphocyte count (p=0.001). It means that patients with CD27 Score 3 have higher lymphocyte count in comparison with those with CD27 score 1.This fact has also been reported by Stefano et al. who also found a significant relation between levels of sCD27 and absolute peripheral blood lymphocytosis ($p < 0.001$), Binet clinical stage ($p = 0.009$), B2M ($p < 0.001$) , sCD23 ($p = 0.0001$), LDH ($p < 0.001$) (26).

All these researches indicate the role of CD27 in clinical severity of CLL. The mechanism that explains that is CD27 expression can be induced by BCR cross-linking or ZAP-70 in CLL patients, so its appearance can reflect recent activation signals that leads to CLL evolvement(10). Most recently constitutive CD27-CD70 signaling have been suggested to stimulate T regulatory lymphocytes which in turn create immuno-suppressive environment for cancer cells (8).

In conclusion, immunohistochemical expression of CD27 in CLL patients directly correlates with advanced stage and increased lymphocytes in blood and BM aspirate. This links between CD27 expression and increased aggressiveness of CLL, and suggests its usage in the predictive plan of CLL patients.

We recommend further prospective studies including larger sample size with follow up of patients to evaluate the relation of immuno-histochemical expression of CD27 to future outcome and chemotherapy responsiveness.

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