Original Article

Evaluation of Bcl 2 and Ki 67 expression in Chronic Lymphocytic Leukemia

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Summary:

Background: Chronic lymphocytic leukemia (CLL) is a low-grade B-lineage lymphoid malignancy. Both Ki-67 which is a large nuclear protein associated with cell proliferation and Bcl-2 which is an anti-apoptotic protein which is associated with dysregulation of the intrinsic apoptotic pathway , were thoroughly investigated in many cancer patients particularly in hemopoietic malignancies .

Patients, materials and methods: This retrospective study was conducted from November 2009 to May 2010 , on fifty formaline fixed paraffin embedded blocks of CLL cases retrieved from Medical City Teaching Hospital ; their age range was 39-75 years along with twenty control cases with benign reactive marrow , The sections of bone marrow biopsies were processed routinely in Al-Kadimiya teaching hospital laboratory and stained with H&E and the expression of Bcl2 and Ki67 was evaluated by light microscope. Smears stained for Bcl2 were classified according to the scoring system used by Soini Y,et al. ,whereas those stained for ki67 were evaluated by estimation the percentage of positive stained nuclei in the smears as was adopted by Diop S,et al.

Results: Immunohistochemical staining of CLL cases revealed that 11/50 cases (22%) were positive for Ki67 showing diffuse or granular nuclear brown stain , whereas all control cases were negative for the marker . Those positive cases significantly correlate with modified Rai system , Hb concentration and platelet count ,but they did not significantly correlate with white blood cell count or lymphocyte % in the bone marrow and peripheral blood smears . Regarding Bcl 2 , 32/50 cases(64%) were positive for the marker showing diffuse cytoplasmic brown reaction ,however no significant correlation was found between Bcl2 score and modified Rai system or with any of the mentioned hematological parameters .Finally , there was no significant correlation between Ki67 and Bcl2 expression .

Conclusions: The proliferative marker Ki-67 expression closely related to the clinical stage of CLL patients , thus it could be considered as an informative and simple tool for assessing disease activity . On the other hand ,although Bcl2 expression was high in CLL patients but it did not related with the clinical stage of those patients . Finally . Ki67 and Bcl2 were independent markers and no correlation was found between them as they act on different pathways since ki67 is a proliferative and Bcl2 is an antiapoptotic marker.

Keywords : Bcl 2 , Ki 67 , CLL.

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Introduction :

Chronic lymphocytic leukemia (CLL) is a distinctive lymphoproliferative disorder characterized by the accumulation of immune-incompetent CD5+ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs . (1,2) According to Iraqi cancer registry (2005) , leukemias rank the fourth of all malignant diseases and CLL ranks the fifth of all leukemias ;its average frequency is 4.36%of all cases of leukemias. (3) In the United States , Australia , Ireland and Italy , chronic lymphocytic leukemia (CLL) is the most common leukemia ; it has an average incidence of 2.7 persons per 100,000 in the United States and ranges from less than 1 to 5.5 per 100,000 persons worldwide. (4)The proto-oncogene bcl2 is a known suppressor of apoptosis , resulting in a long life for the involved cells. (5,6) It was discovered by virtue of its activation by the t(14:18) translocation in the majority of follicular B-cell lymphomas (FL) .This translocation fuses the Bcl-2 open reading frame with immunoglobulin regulatory sequences, causing over expression of the intact Bcl-2 protein .(6) Due to the key role of apoptosis in the progression of B-CLL, multiple studies have investigated Bcl-2 protein expression and regulation of BCL2 gene transcription .(7,8) The Ki-67 gene is located on the long arm of human chromosome 10 (10q25) .(9) It code for a large nuclear protein associated with cell proliferation thus it is absent during the G0 phase. It is well know that the vast majority of the circulating B-CLL cells are arrested in G_0/G_1 phase of the cell cycle . However, isotopic labeling of leukemic cells in vivo revealed that a substantial fraction of the B-CLL cells does proliferate and generate new cells which takes place in so-called proliferation centers frequently found in lymph nodes and bone marrow of patients with B-CLL. Since Ki-67 antigen reflects the status of proliferation activity of the cells thus, it was predicted that the

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level of its expression in advanced stage of CLL was higher than that in early stage of CLL. (10) These new insights on the proliferation pathways in CLL not only may provide a better understanding of the pathogenesis of this disease, but also would be of prognostic relevance and can support the use of new treatments aimed at inhibiting proliferation in CLL. (11)

Patients , Materials and methods :

This retrospective study was conducted from November 2009 to May 2010 on fifty patients with chronic lymphocytic leukemia. The cases were retrieved from archive files of the Department of Hematology of the Medical City Teaching Laboratories . Patients were randomly selected regarding age and sex and were newly diagnosed and were not on any medication prior to the bone marrow biopsies. Clinical and laboratory information regarding age ,sex, PCV, WBC count, platelet count, percentage of lymphocyte in the bone marrow and the pattern of bone marrow infiltration were obtained from patients recording file at diagnosis .Modified Rai staging system was adopted for staging of patients with CLL accordingly they were classified into three groups: low, intermediate and high risk groups Three sections were taken from each formalin fixed paraffin embedded bone marrow of MM and of control cases . At Al Kadimiya teaching hospital lab, one section was stained with hematoxylin and eosin and two other sections were stained immunohistochemically for Bcl2 and Ki67 with horseradish peroxidase (HRP)-labelled –streptavidin –biotin method . The slides were examined by light microscope and scanned on low and high power (10x40x). This technique basically uses an unlabeled primary antibody, which was mouse monoclonal antibody purchased from DAKO (code no. of the kits was M7240 for Ki67 and M0887 for Bcl2) , it binds to its corresponding antigen, followed by a biotinylated secondary antibody to which the avidin-biotin complex (one avidin molecule, three biotin-labeled peroxidase molecules) attaches. If the sought-after antigen is present in the section, there will be an antibody-antigen interaction and an enzymatic reaction that can be detected by the chromogen, diaminobenzidine (DAB), which can be visualized by light microscopy .Negative controls were obtained by replacing the primary antibody with buffer saline and positive controls for each antibody were included with the samples using follicular lymphoma for Bcl2 and reactive lymphoid hyperplasia for Ki 67.

Results :

In this study the age of patients ranged between 39-75 years with a mean=59.2±1.34 (Mean ± SE) years and 56% of the cases were above 60 year. Figure 1

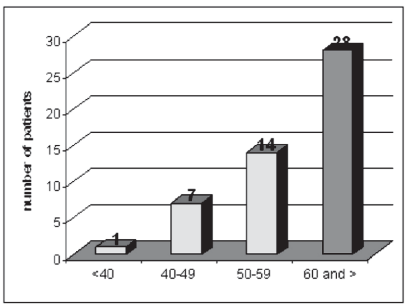

Figure 3.1: The distribution of CLL patients according to agegroups

Regarding the gender of the patients , 38 (76%) were males and 12 patients (24%) were female. The most common presenting features were lymphadenopathy (72%) followed by splenomegaly (48%), fever (10%) and hepatomegaly (8%) .Regarding the hematological parameters ,the Mean± SEM of Hb level was 10.52 ±0.26 g\dl , of platelet count was 142.82±10.11 x10^9\L and of absolute lymphocyte count was 110.97±14.40 x10^9\L. Of all patients one patient had positive direct antiglobulin test (DAT).By applying the Modified Rai staging System , the studied patients were categorized into the following:

1- 27patients (54%) were in the high risk group .17 patients (34%) were in the intermediate risk group.6 patients (12%) were in the low risk group . 3-

Regarding bone Marrow histology_diffuse involvement of BM by CLL was found in 31 cases (62%), interstitial in 11 cases (22%) and mixed involvement in 8 cases(16%).

By applying chi-square test there was significant association between the bone marrow patterns and the clinical stages presented as modified Rai staging system (P= 0.006)as shown in table 1.

Table 1 : The association between BM histology and modified Rai staging

		BM histology			
		Diffuse	Interstitial	Mixed	Total
Modified Rai Stage	High risk	17	5	5	27
	Inter mediate risk	13	1	3	17
	Low risk	1	5	0	6
	Total	31	11	8	50

The expression of Ki 67 appears as nuclear brown stain which may be diffuse , granular, or a combination (Figure 2) as was detected by other worker . (12) Diop S, et al (13) scoring system was adopted for evaluation of Ki67 staining which was based on counting the percentage of positively stained nuclei . Accordingly 39 cases (78%) were negative and 11 cases (22%) were positive and the score distribution for positive cases was shown in table 2 .

Table 2 : ki-67 score distribution for positive cases with CLL

Ki-67 score	Number of cases	Percent
≤ 2%	5	10.0
3-9%	4	8.0
≥10%	2	4.0
Total	11	22.0

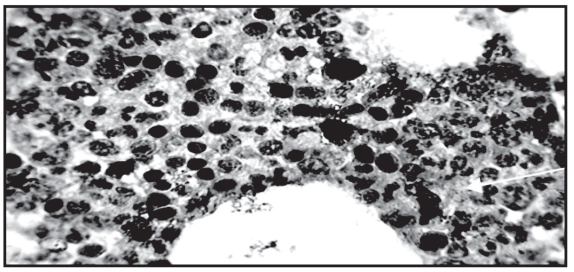

Figure 2:Bone marrow from CLL patient stained with Ki67(X100) (Pink arrow=diffuse pattern ,white arrow =granular pattern)

By applying chi square, there was no significant association between Ki-67 score and age (P= 0.115) , gender (P=0.865) , lymph node enlargement (P= 0.385) or BM histology (p=0.611) . However there was a significant association between ki-67 score and splenomegaly(P=0.013).

Moreover there was significant inverse correlation between Ki67 score and Hb concentration (p=0.003) and platelet count (p=0.039) ,but there was no significant correlation between ki-67 score and WBC count or lymphocyte % in the bone marrow or peripheral blood. (Table 3)

Table 3 : The correlation between Ki 67 score and hematological parameters .

	Ki-67 score	N	Mean	SEM	Minimum	Maximum	p-value
WBC count ($x10^9$/L)	0%	39	111.1667	17.11202	12.00	487.00	0.327
	≤ 2%	5	140.8000	30.49328	72.00	220.00	
	3-9%	4	204.5000	59.31062	62.00	339.00	
	≥10%	2	72.5000	29.50000	43.00	102.00	
Hb (g/dl)	0%	39	10.5897	.28269	6.50	14.00	0.003*
	≤ 2%	5	12.4000	.53385	11.00	14.00	
	3-9%	4	9.0000	.35355	8.00	9.50	
	≥10%	2	7.5000	1.00000	6.50	8.50	
Platelet count ($x10^9$/L)	0%	39	150.7949	11.68786	18.00	380.00	0.039*
	≤ 2%	5	171.6000	15.01533	140.00	224.00	
	3-9%	4	72.0000	14.39329	29.00	90.00	
	≥10%	2	57.0000	3.00000	54.00	60.00	
LC % in BM (%)	0%	39	84.92	1.812	50	99	0.604
	≤ 2%	5	83.80	1.715	80	89	
	3-9%	4	90.00	3.536	80	95	
	≥10%	2	92.50	2.500	90	95	
LC % in blood (%)	0%	39	88.28	1.36	61.00	99.00	0.119
	≤ 2%	5	86.40	3.93	73.00	96.00	
	3-9%	4	98.50	.64	97.00	100.00	
	≥10%	2	90.50	5.50	85.00	96.00	

*=significant correlation

There was significant association between ki-67 score and Modified Rai staging (P= 0.042) as shown in table 4 :

Table 4 : The association between ki-67 score and Modified Rai stage

		Ki67score				
		0%	≤ 2%	3-9%	≥10%	Total
Modified Rai stage	High risk	21	0	4	2	27
	Intermediate	13	4	0	0	17
	Low risk	5	1	0	0	6
	Total	39	5	4	2	50

P= 0.042 (S) (Chi-square)

Regarding Bcl2 expression the presence of diffuse cytoplasmic brown reaction is indicative of positive reactivity (Figure 3) , which is similar to the result of other workers (7,14)as shown in figure 2. The scoring system used by Soini Y,et al was adopted for classification of the cases (15); accordingly 18 cases (36%) were negative and 32 cases(64%) were positive for Bcl2 and the score distribution for
positive cases is shown in table 5.

Table 5 : Bcl2 score distribution for positive cases

Bcl2 score	Number of cases	Percent
+(weak)	16	32.0
++(moderate)	7	14.0
+++(strong)	2	4.0
++++(very strong)	7	14.0
Total	32	64.0

The staining character and localization is shown in the following figures:

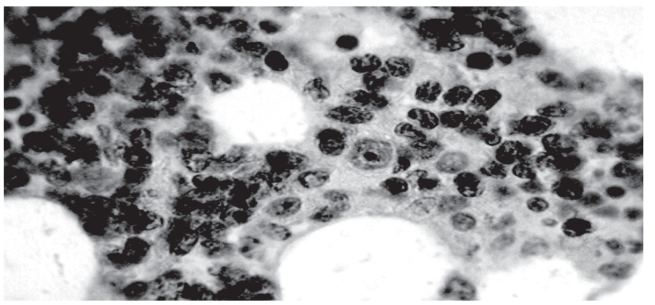

Figure 3 : Bone Marrow tissue from CLL patient stained with anti Bcl2 (100x)

By using Chi square , there was no significant association between Bcl2 score and age (p= 0.497) , gender (P= 0.464) , lymphadenopathy (p=0.523), splenomegaly (p=0.488),pattern of bone marrow histology (P=0.356) or Modified Rai stage (p=0.414) . There was no significant correlation between Bcl2 score and hematological parameters including WBC count (p=0.217) ,Hb concentration (p=0.492). ,Platelet count (p=0.489), lymphocyte % in the bone marrow (p=0.461)or **peripheral blood (p=0.249).**

Table 6 : The association between Bcl2 score and Modified Rai stage

		BCL2 score					
		zero	Weak	moderate	strong	very strong	Total
Modified Rai stage	high risk	10	8	5	2	2	27
	intermediate risk	6	5	1	0	5	17
	low risk	2	3	1	0	0	6
	Total	18	16	7	2	7	50

*P= 0.414 (NS)

Finally by applying spearman rank linear correlation , there was no statistically significant correlation between Ki67 and Bcl2 expression (p=0.532) .

Discussion :

In this study the age of the patients and the male/female (M: F) ratio were comparable to that reported by other Iraqi workers (14,16,17) ; but the age rang was lower and the M/F ratio was higher than that reported in various western studies (2,18) This difference can be attributed to the variation in population structure , as life expectancy in Western countries is longer than in Iraq , lack of routine checkup for Iraqi patients and difference in the sample sizeSimilar to many Iraqi studies(14,16) , and Western studies (2,18) the incidence of lymphadenopathy was more common than splenomegaly and hepatomegaly Moreover, only one patient (2%) was asymptomatic at diagnosis similar to other Iraqi studies (14,16,17), but differ from Western studies (17,19) which showed that approximately 50% of patients with CLL were asymptomatic at diagnosis .This may be attributed to lack of routine checkup for Iraqi patients thus, they will presented in advanced clinical stage .This concept was further confirmed by observing the distribution of cases in relation to the modified Rai classification which revealed that 54% of the patients were in high-risk stage, 34% in intermediate stage and only 12% were in low-risk stage , whereas in Western countries 40 -60% of CLL patients were in the early clinical stage of the disease at the time of diagnosis (17).Moreover 62% of CLL patients presented with diffuse pattern of bone marrow infiltration unlike Western countries in which non-diffuse histological patterns predominated and the diffuse pattern was found in only 9.8% (20) This study revealed that Ki67 was not express in 78% of cases and since virtually all circulating B-CLL lymphocytes were long-lived elements arrested in the G0/early G1 phase of the cell cycle while Ki67 is associated with cell proliferation and its level is low during G1- and early S-phase and progressively increase to reach a maximum during mitosis and it cannot be detected in G0 quiescent cells (21), thus most of CLL lymphocytes did not express the marker . However the highest expression was detected in high risk patients of the modified Rai system whereas the intermediate and low risk group showed low expression of the marker , this due to that high risk patients may have more active disease with focal aggregates of proliferating cells and those cells are expected to show high expression of Ki67 . Those results were in agreement with Quijano S, et al who found that high proliferative rate in B-cell chronic lymphoproliferative disorders usually associated with high Ki67 expression , typically ≥ 10% and they run an aggressive and progressive clinical course , and a shorter overall survival (22). Moreover Diop S,et al study found that the proportion of prolymphocyte in the peripheral blood which is a known bad prognostic parameter(1) correlates well with the percentage of Ki-67+ leukemic cells(13) ,and that the large lymphoma cells of Richter's syndrome were positive for Ki-67 (23). Thus we may conclude that Ki67 can be used as a marker for the activity of the disease .Moreover ,in the current study , Ki67 score correlate well with Hb concentration and platelet count but not with WBC count and lymphocyte percent which clarify that Ki 67 correlate with the activity of the disease rather than with the quiescent cells count Regarding Bcl 2 , although it was express in 62% of cases however there was no significant association between Bcl-2 score and clinical features of the patients , modified Rai stages of the disease or other hematological parameters. Similar results were obtained in Iraq by Abdulkareem, et al who had evaluated Bcl2 by In Situ Hybridization method. Similar results was reported by Marschitz, et al. study, who had used immuno-cytochemical method rather than immunohistochemstry to analyze Bcl-2 protein expression in CLL (7,16). The high levels of Bcl-2 protein expression in CLL cases, was hypothesized to reflect an intrinsic abnormality, akin to that seen in follicular lymphoma(24).However, recent studies revealed that the Bcl-2expression in CLL is not autonomous and it may be affected by external stimuli , by microenvironment or in response to certain drugs and it may not play a major role in the pathogenesis of the disease (25,26).In addition, Schimer, et al and Del Gaizo, et al had concluded that although Bcl-2 expression was associated with a poor response to cytotoxic therapy for CLL, but it does not appear to be a major determinant of clinical progression (27,28). Finally, there was no significant correlation between Ki67 and Bcl2 score. This may be explained by the fact that those two markers act on different pathway and they were independent on each other, since ki67 is a proliferative antigen whereas Bcl2 is an anti apoptotic protein . Thus we may conclude that Ki-67 expression is closely related to the clinical stage of the patient , thus it can be considered as informative and simple tool for assessing disease activity. On the other hand , although Bcl2 expression is high but it did not correlate with the clinical stage of the patient . Finally no correlation was found between Ki67 and Bcl2 expression as they act on different pathways since ki67 is a proliferative and Bcl2 is an anti apoptotic marker.

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