ZAP 70 expression in bone marrow biopsy of Chronic Lymphocytic Leukemia among Iraqi people

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<u>الخلاصة</u>
<u>خلفية :</u>

مرض ابيضاض الدم الليمفاوي المزمن (CLL) هو سرطان مزمن للخلايا اللمفاوية نوع بي. يتميز بصفات مظهرية مجهرية و علامات سرطانية مناعية خاصة . وهو مرض متغير السريرية وهناك عدد من العلامات المناعية تستخدم لتقيم نشاط المرض و ما ستؤول إلية الحالة المرضية .

CD5 هو سلسلة أحادية من الكلايكوبروتين بوزن ٦٧ كيلو دالتون . الأجسام المضادة لـ CD5 تستعمل كوسيلة مهمة لتشخيص و تصنيف السرطانات اللمفاوية .

ZAP 70 هو بروتين بوزن ٧٠ كيلو دالتون. وهو احد عناصر عائلة بروتينات التايروسين كاينيز. ويعتبر علامة مميزة لحدوث طفرة وراثية في ((IgV(H) وبذلك يكون من المعلمات السرطانية المهمة لتحديد ما ستؤول إلية حالة المرضى المصابين بأبيضاض الدم اللمفاوي المزمن .

أهداف الدراسة:

١- معرفة أكثر العلامات السريرية و المختبرية شيوعا لمرضى ابيضاض الدم اللمفاوي المزمن.

٢- تقييم التعبير الكيميائي المناعي لكل من (CD5 و ZAP70) في خزعة نخاع العظم لمرضى ابيضاض الدم اللمفاوي المزمن.

٣- معرفة أهمية ظهور التعبير الكيميائي لـ ZAP 70 في خزعة نخاع العظم لمرضى ابيضاض الدم اللمفاوي المزمن.

المرضى والمواد والطرق :

هذه الدراسة تمت بأثر رجعي على أربعين عينة من خزعة نخاع العظم المحفوظة في مادة البرافين الشمعية لأربعين مريض مصاب بمرض ابيضاض الدم اللمفاوي المزمن جُمعت من المختبرات التعليمية في مدينة الطب في بغداد جنب إلى جنب مع عشر خزعات مراقبة لنخاع العظم من أشخاص طبيعيين خلال الفترة المحصورة بين تشرين الاول ٢٠١٠ و نيسان ٢٠١١ .

المقاطع المأخوذة من خزع نخاع العظم عولجت بصورة روتينية بالاصطباغ المناعى الكيميائي لكل من (CD5 و ZAP70) و تم فحصها بواسطة المجهر الضوئي.

الصبغة المناعية لـ CD5 قد صنفت وفقا لنظام التسجيل التي يستخدمها *Dorfman DM ,et al.* في حين استند تقييم الصبغة المناعية لـ ZAP70 على ما تم استعمالة من قبل *Wiestner A ,et al.*

النتائج :

أهم العلامات السريرية كانت اعتلال لمفي غدي ، تضخم الطحال ، تضخم الكبد ، فقدان الوزن و الحمى وبصورة متتالية .

أهم الموجودات المختبرية كانت زيادة في كريات الدم البيض ، زيادة مطلقة بالخلايا اللمفية ، فقر الدم و نقصان الصفيحات الدموية . وكانت اغلب الحالات المرضية تقع ضمن التدرج عالي الخطورة لنظام التصنيف Modified Rai.

التعبير المناعي الكيميائي لـ CD5 وجد في جميع الحالات المرضية لأبيضاض الدم اللمفاوي المزمن و بذلك ليس له أي دالة إحصائية لنشاط الحالة المرضية و ما ستؤول إلية .

هذه الدراسة أظهرت صلة إحصائية وثيقة بين ظهور التعبير المناعي الكيميائي لـ ZAP70 و التدرج السريري بواسطة Modified Rai (P = 0. 05) .

وعلاوة على ذلك كانت هناك علاقة عكسية ذات دلالة إحصائية بين التعبير المناعي الكيميائي لـ ZAP70 مع نسبة الـ PCV (P=0.05) وعدد الصفائح الدموية (P=0.001) في حين لا يوجد ترابط إحصائي بين التعبير المناعي الكيميائي لـ ZAP70 و عدد كريات الدم البيض أو نسبة الخلايا اللمفية في الدم ونخاع العظم.

وكذلك ، كشفت هذه الدراسة أنه ليس هناك ارتباط إحصائي بين علامات (CD5 و ZAP70).

Abstract:

Background: Chronic lymphocytic leukemia (CLL) is a chronic B-lineage lymphoproliferative disorder characterized by specific morphological and immunophinotyping features. It has a variable clinical course and several biological markers which were used to assess the disease activity and prognosis .

Objective: This was designed to determine the frequency of immunohistochemical expression of CD5 and ZAP 70 (zeta associated protein of 70 kDa)in bone marrow biopsy of CLL patients and detect the significance of ZAP 70 immunohistochemical expression in bone marrow biopsy as prognostic factor .

Patients and methods: This retrospective cross-sectional study is conducted on forty formalin fixed paraffin embedded blocks of chronic lymocytic leukemia cases collected from teaching laboratories of medical city in Baghdad along with ten control cases of normal bone marrow during October 2010 through April 2011.The sections of bone marrow biopsies were processed routinely with immunohistochemical stain for CD 5 and ZAP 70 and examined by light microscope.

Results: Main clinical findings of CLL cases were lymphadenopathy, splenomegaly, hepatomegaly , weight loss and fever respectively while asymptomatic cases constitute only 10 % of the cases . Main laboratory findings were leucocytosis , absolute lymphocytosis , anaemia and thrombocytopenia . Most of these cases were within the high risk group by use modified Rai staging system .CD 5 expression was found in all cases. So, there is no statistical significant association with disease activity or prognosis, while ZAP 70 expressed in 48 % of study cases and our study revealed that there was a statistical significant association between ZAP 70 expression and the clinical stage of the disease using Modified Rai system . Moreover ,there was a statistical significant inverse association between ZAP 70 expression with PCV % and platelet count. Whereas no statistical significant association between ZAP 70 expression and white blood cell count , lymphocyte % in the Bone Marrow and peripheral blood or bone marrow involvement by lymphocytes .

Conclusions: This study revealed that CD 5 is expressed nearly in all cases of CLL and gives information that this disease is mostly a B cell linage neoplasm and carry's no significance with disease progression, ZAP 70 is considered an important prognostic marker in CLL and closely related to clinical stage of the disease. Therefore it can predict a clinical course in CLL , ZAP 70 positive score is in significant association with advanced stage of CLL and usually with worse outcome and there is no significant association between CD 5 and ZAP 70 expression in CLL cases was noted.

Introduction: Chronic lymphocytic leukemia (CLL) is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes in peripheral blood, bone marrow and lymphoid organs[1]. It has been known that the clinical course of CLL is variable, with some patients experiencing indolent disease not requiring any therapy, whereas others demonstrate a more aggressive course, unresponsive to therapy, with substantially shorter survival[3]. In B-cell chronic lymphocytic leukemia , lymphocytes typically show B-cell surface antigens as demonstrated by CD19, CD20, CD21, and CD23 monoclonal antibodies. In addition, they express CD5 which is more typically found on normal T and subset of normal B cells [4,5].

CD5 is present in nearly all cases of CLL, SLL and the great majority of MCLs. It is generally absent in other B-cell lymphomas [6] .

ZAP-70 gene codes for a 70 KDa tyrosine kinase that is normally expressed in T-cells and NK cells. In the June 2003, Wiestner and colleagues report that increased

expression of the ZAP-70 gene (as determined by quantitative analysis of ZAP-70 mRNA) correlates well with unmutated IgVH status in patients with CLL. Furthermore, they found that the expression of the ZAP-70 gene protein (assessed by immunehistochemistry) was also found to have a good correlation with IgVH mutation status [7]. Our trial was designed to estimate the rate of immunohistochemical expression of ZAP 70 in our country among CLL patient and to study its role in CLL prognosis .

Materials and methods: **Study group** included forty cases (31 males and 9 females) of CLL with age range from 34 to 75 years. Relevant information were noted on prepared sheet including age, gender, clinical findings (lymphadenopathy , splenomegaly , hepatomegaly , pallor , bleeding , infection , weight loss and other) and laboratory findings (CBP , peripheral blood , bone marrow aspirate and biopsy) .

   Control and comparative group: Ten cases (7 male and 3 female) of normal bone marrow biopsy were selected their age range from 35 to 70 years . Each step done for the control group was in parallel with the study group.

   Positive control slides : Parallel positive control sections were processed with each set of immunostaining. Positive controls of tonsil tissue involved by benign reactive inflammation which is known to express CD 5 and ZAP 70 [8] , respectively were used with each run .

   Negative control slides: Normal bone marrow biopsies were taken as negative control in each run [8].

   Immunohistochemical staining protocol : The immunostaining method used in the current study was Labelled Strept - Avidin Biotin (LSAB+) technique which was applied for both CD5 and ZAP 70 staining [17]:

   Evaluation of all the study cases including :

1. Re evaluation of all available slides of peripheral blood smear and bone marrow aspirate and biopsy for CLL cases and assess with their related notes of diagnosis .

2. From bone marrow biopsy of each case two sections had been taken and were stained immunohistochemically for CD 5 and ZAP 70 . The slides were examined by light microscope and scanned on low and high power (10x,40x and 100x).

3. 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.(Low-risk patients: With only lymphocytosis in the PB and BM , Intermediate-risk patients: With lymphocytosis and lymphadenopathy and/or hepatosplenomegaly and High-risk patients: With lymphocytosis together with anemia (Hb < 11 g/dL) (stage III) and/or thrombocytopenia [18].

Scoring system: The scoring system for CD 5 were scored positive if 50% or more of the cells within an aggregate showed cellular membrane and/or cytoplasmic staining pattern [5] **, while ,** the scoring system for ZAP 70 were scored positive if 20% and more of the cells within an aggregate showed nuclear and cytoplasmic stain [9,10,11] .

   Statistical analysis was performed with **SPSS** 13 (statistical package for social sciences) and **Excel 2003** programs. Data analysis was done using t- test, analysis of variance (ANOVA) and chi –square test for tables with frequencies, percentages, range mean and standard deviation, P value was (0.05).

Results : The maximum age group (45%) were within the 7th decade of life and more, while (35%) were within the 6th decade , (17.5%) within the 5th decade and only (2%) was within the 4th decade. In our study male constitute 77.5% (31/40) of patient and female were 22.5 % (9/40) so, male to female ratio was about 3.4:1. The most common clinical features of CLL cases in this study were lymphadenopathy (72.5%) followed

by splenomegaly (45%), hepatomegaly (22.5%) , weight loss (20 %) and fever (17.5 %).

Regarding to clinical stage (52.5%) were in the high risk group , (30 %) were in the intermediate risk group and (17.5%) were in the low risk group by use modified Rai staging system .

Hematological parameters in study group : The mean PCV percent was were less than normal range to age and gender in 43% of cases. The platelet count was less than normal range in 50% of cases . The WBC count was more than normal range in 95% of cases . The lymphocyte percent in peripheral blood was (89.4 $\pm$ 9.3) % of circulating white blood cells and majority with typical mature looking lymphocytes . The mean lymphocyte percent in bone marrow aspirate was (85.6 $\pm$ 13) % of all nucleated cells . Bone marrow histological lymphocytes involvement was diffuse pattern in 55% of CLL cases , interstitial in 30% of cases and mixed in 15% of cases.

There was a statistical significant positive association between Bone Marrow percentage of lymphocytes and peripheral blood lymphocytosis while, there was a statistical significant inverse association between lymphocyte % in the Bone Marrow and PCV % and platelet count . as in table 1 :

Table 1 The hematological parameters of patients included in the study (N=40)

	Range	Mean	SD
PCV %	21%- 46%	34.67 %	6.90
Platelet count(x10^9\L)	29-320	136.57	75.37
*WBC count	6.8-458	102.34	97.75
**L.C. (%) in peripheral blood	61% -100%	89.4	9.35
**L.C. (%) in bone marrow aspirate	40 % - 99 %	85.60	13.04

Immunohistochemical results: 40 cases (100 %) were positive for CD5 as shown in table 1.

Table1. CD5 expression in CLL cases

CD 5 expression	Number of cases	Percent
Positive	40/40	100 %
Negative	Zero/40	Zero %
Total	40	100%

The staining character and localization of CD 5 in CLL and control group in our study are shown in figure 1 :
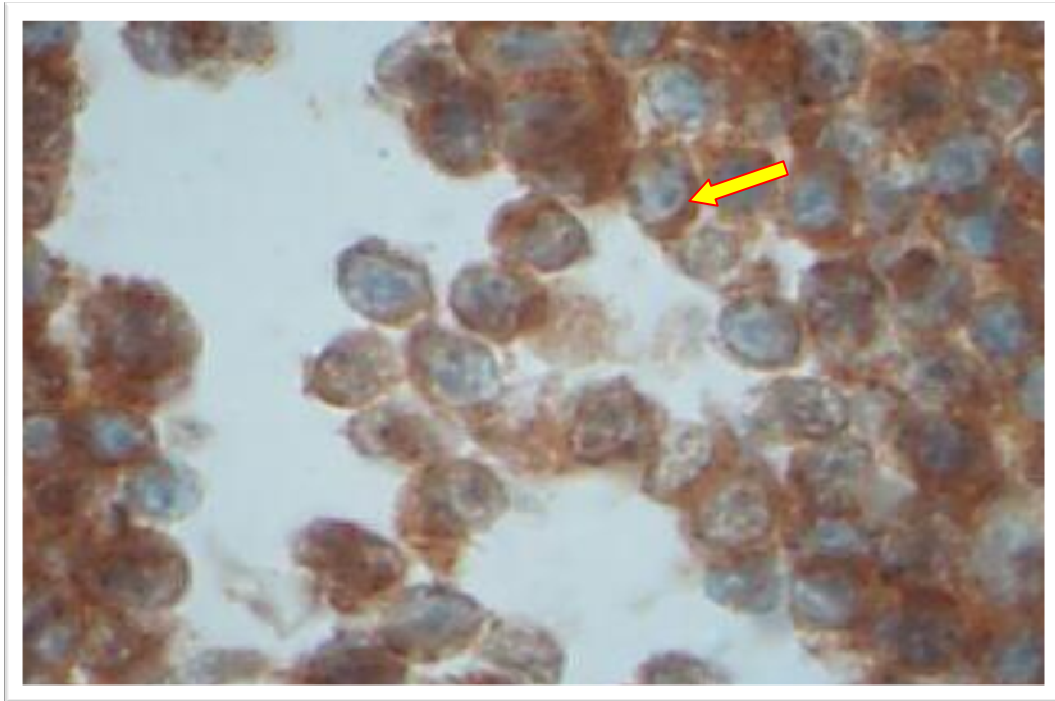
Figure 1 bone marrow tissue of CLL stained with CD 5 most of cells show brown membrane and cytoplasmic stain (CD5 positive) (x 100).

ZAP 70 expression : 19 cases (48%) were positive for ZAP 70 and 21 cases (52%) were negative for ZAP 70 among CLL cases while, all control group were negative and the score distribution for CLL cases is shown in table 2 :

Table 2 : ZAP 70 score distribution for CLL cases .

ZAP 70 score	Number of cases	Percent
Negative	21/40	52 %
Positive	19/40	48 %
Total	40	100 %

The staining character and localization of ZAP 70 in CLL and control group in our study are shown in figure 2 :
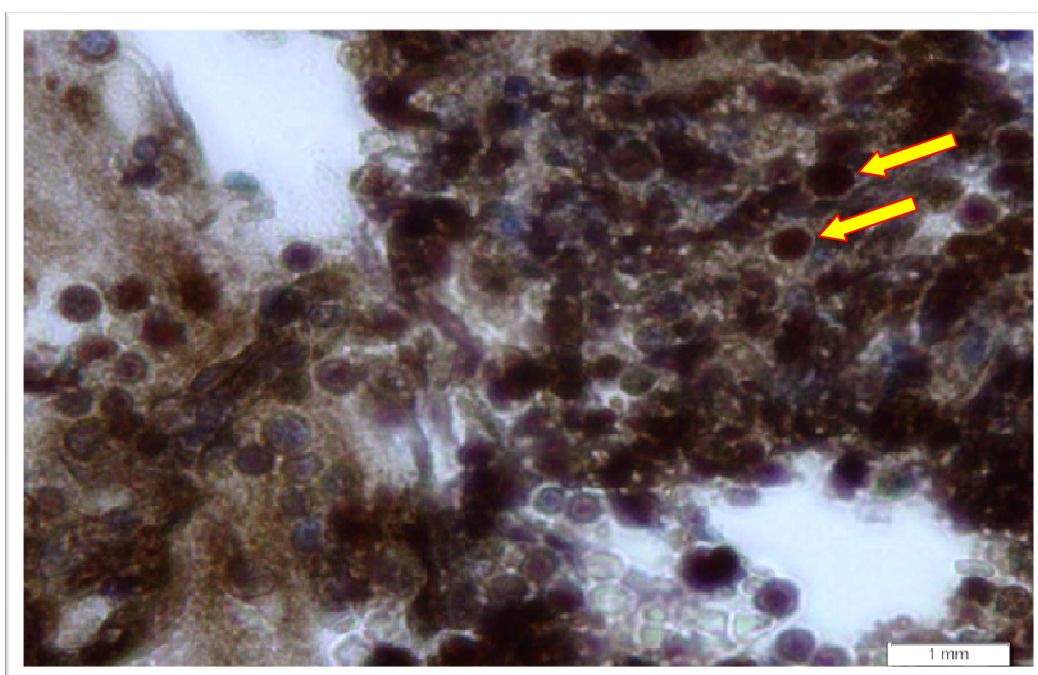
Figure 2 bone marrow tissue of CLL stained with ZAP 70 more than 20% of cells show brown nuclear and cytoplasmic stain (Zap 70 positive) (x 100).

There was a statistical significant association between ZAP 70 expression and Modified Rai staging in CLL cases. Also, There was a statistical significant inverse association between ZAP 70 expression and PCV percent and platelet count, but there was no statistical significant association between ZAP 70 expression and WBC count , lymphocyte percent or morphology in the peripheral   blood or bone marrow aspirate as shown in table 3 using t. test.

Table 3 :The correlation between ZAP 70 expression and hematological parameters

	ZAP 70 score	N	Mean	SD	Minimum	Maximum	p-value
Platelet count (x10^9/L)	Negative	21	156.571	63.3	34.00	320.00	0.05 Inverse association
	positive	19	119.157	35.6	29.00	320.00	
PCV (%)	Negative	21	38.04	6.06	22.00	46.00	0.001 Inverse association
	positive	19	31.31	5.49	21.00	40.00	
WBC count (x10^9/L)	Negative	21	80.061	68.4	12.00	252.00	0.05 No ass0ciation
	positive	19	127.163	119.	6.8	458.00	
LC % in peripheral blood	Negative	21	88.04	10.1	61.00	99	0.05 No association
	positive	19	90.89	8.44	73.00	100	
LC % in BM aspirate	Negative	21	82.333	15.6	40	96	0.05 No association
	positive	19	89.210	8.35	66	99	

Discussion : CD5 is present on nearly all cases of CLL/SLL, and the great majority of MCLs. It is generally absent in other B-cell lymphomas (6) . In our study, CD 5 was 100% positive in study group while in all of control group it is found 100% negative . Accordingly , it carries no significance in this study behind the confirmation of B-cell CLL diagnosis .

ZAP-70 expression correlates with immunoglobulin heavy-chain variable region gene mutational status in CLL/SLL, and can be detected reliably using immunohistochemical methods (13) . In our study the frequency of ZAP 70 positive score was 48% , whereas the control group were negative for ZAP 70 totally.

Similar results were reported by Cameron Yin *et al* and Marjan Ertault , Maria Elena Noguera, *et al*. who had use immunophinotyping study of ZAP 70 expression in bone marrow of CLL cases by flowcytometry technique [14,15] . In addition , our results were slightly more than Michele; Rachel *et. al.* who found (41%) of CLL cases in their study were positive for ZAP 70 by immunohistochemistry technique [16]. The present study revealed that there was no significant association between ZAP 70 score with gender , age , clinical presentation or bone marrow histology of patient . Nevertheless there was a significance association with modified Rai staging system exhibited in the large percent (73.68 %) of positive ZAP 70 group with high risk group , (21.05 %) with intermediate risk group and (5.26 %) with low risk group . This significant assiciation reflects the importance of ZAP 70 as a prognostic marker in CLL.

There was a significant inverse association between ZAP 70 expression and PCV percent and platelet count, but there was no significant correlation between ZAP 70 score and WBC count, morphology or lymphocyte % in the bone marrow or peripheral blood .

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