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Correlation of c-MYC, BCL2 and BCL6 proteins overexpression with prognosis in Syrian DLBCL Patients

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Abstract:

Background and Aim: c-MYC is a potent transcription factor involved in several cellular processes, and is overexpressed in many cancers, including non-Hodgkin lymphoma (NHL). The most common subtype of NHL is diffuse large B-cell lymphoma (DLBCL). DLBCL tumors expressing both the c-MYC and BCL2 proteins, i.e. double expressor (DE), also triple expressors (TE) expressing BCL6 in addition to c-MYC and BCL2, are all more aggressive and refractory to treatment.

Our study aims to investigate auxiliary immunohistochemical tests (IHC) not routinely used, namely c-MYC, BCL2 and BCL6, seeking a better prognostic testing protocol for DLBCL patients.

Materials and Methods: Forty-one formalin-fixed paraffin-embedded (FFPE) DLBCL samples were collected retrospectively from the pathology laboratory at Al-Assad University Hospital after approval from the ethics committee at Damascus University. Clinical data was obtained from patients records in regards to progression-free survival (PFS) and other parameters.

Slides were microtomed from each FFPE sample and used to perform IHC tests of the c-MYC, BCL2 and BCL6 oncoproteins. Fisher's exact test was used to study the independence of protein IHC results in relation to one another and compared with PFS.

Results: Statistical analysis of the c-MYC overexpression and double expressor status showed no correlation ($P>0.05$) with PFS in DLBCL patients. Whereas, when BCL6 IHC results were analyzed, BCL6 positivity and triple expressor status showed a significant association ($P<0.05$) with unfavorable PFS outcomes, where BCL6 positive patients were three times more likely to progress to an unfavorable outcome in comparison to BCL6 negative patients within three years of diagnosis

Conclusions: For the determination of DLBCL prognosis, routine IHC testing for BCL6 protein overexpression is recommended.

Thus, we suggest that BCL6 IHC testing should be added to the workup protocol of DLBCL patients in Syria.

Keywords: IHC, DLBCL, C-MYC, BCL2, BCL6, Double Expressor, Triple Expressor.

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ارتباط فرط التعبير عن البروتينات c-MYC وBCL2 وBCL6 مع الإنذار عند مرضى لمفومة الكريات البائية الكبيرة المنتشرة السوريين

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الملخص:

خلفية البحث وأهدافه: يُعد c-MYC عامل انتساخ قوي يُسهم في العديد من العمليات الخلوية، كما يتم التعبير عنه بشكل فائض في العديد من السرطانات ومن ضمنها اللمفومة اللاهودجكينية. تُعتبر لمفومة الكريات البائية الكبيرة المنتشرة نُمَيط اللمفومة اللاهودجكينية الأكثر انتشاراً. وتكون أورام هذه اللمفومة المُعبرة عن البروتينين c-MYC وBCL2، أي مزدوجة التعبير، أوالأورام ثلاثية التعبير التي تعبر أيضاً عن BCL6 بالإضافة إلى c-MYC وBCL2، جميعها أكثر عدوانية ومقومة للعلاج.

يهدف بحثنا إلى التحرّي عن اختبارات كيميائية هيستولوجية مناعية مُساعدة غير مستخدمة روتينياً، وهي c-MYC وBCL2 وBCL6، التماساً لبروتوكول اختبارات إنذارية أفضل لمرضى لمفومة الكريات البائية الكبيرة المنتشرة.

مواد البحث وطرائقه: جُمعت 41 عينة لمفومة كريات بائية كبيرة منتشرة مثبتة بالفورمالين محفوظة في البارافين بشكل استعادي من مختبر الباثولوجيا في مستشفى الأسد الجامعي بعد الحصول على موافقة اللجنة الأخلاقية في جامعة دمشق. جُمعت المعلومات السريرية من سجلات المرضى فيما يتعلق بالبقيا دون تفاقم وغيرها من المعالم.

اقتُطعت شرائح من كل عينة واستُخدمت لإجراء اختبارات الكيمياء الهيستولوجية المناعية للبروتينات الورمية c-MYC وBCL2 وBCL6. استخدم اختبار فيشر الدقيق لدراسة استقلال نتائج الكيمياء الهيستولوجية المناعية للبروتينات المدروسة وعلاقتها ببعضها البعض وبالمقارنة مع البقيا دون تفاقم.

النتائج: لم يُظهر التحليل الإحصائي لفرط التعبير عن c-MYC وحالة التعبير المزدوج علاقة ارتباط ($P>0.05$) مع البقيا دون تفاقم لدى مرضى لمفومة الكرات البائية الكبيرة المنتشرة. بينما أظهرت نتائج الاختبارات الهيستولوجية المناعية الإيجابية لبروتين BCL6 والحالة ثلاثية التعبير ارتباطاً يُعتد به إحصائياً ($P<0.05$) مع نتائج ضائرة للبقيا، حيث كان المرضى إيجابيي BCL6 أكثر احتمالية لتفاقم المرض أو الوفاة بثلاث مرات مقارنة بالمرضى سلبيي BCL6، وذلك خلال السنوات الثلاث الأولى بعد التشخيص.

الاستنتاجات: يوصى باعتماد اختبارات الكيمياء الهيستولوجية المناعية لكشف فرط التعبير عن بروتين BCL6 روتينياً لدى مرضى لمفومة الكريات البائية الكبيرة المنتشرة وذلك لتعيين الإنذار.

وعليه نقترح إضافة اختبار BCL6 بطُرُق الكيمياء الهيستولوجية المناعية إلى بروتوكول إجراءات التشخيص لمرضى لمفومة الكريات البائية الكبيرة المنتشرة في سورية.

الكلمات المفتاحية: الكيمياء الهيستولوجية المناعية، لمفومة الكريات البائية الكبيرة المنتشرة، c-MYC، BCL2، BCL6، مزدوج التعبير، ثلاثي التعبير.

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Introduction:

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of hematological malignancies encompassing several subtypes categorized based on the origin of tumor cells; B lymphocytes, T lymphocytes, or natural killer (NK) cells. Each subtype is further divided into entities according to their morphological and clinical presentations[1].

The most common NHL subtype -accounting for 30-40% of cases- is diffuse large B-cell lymphoma (DLBCL); in itself a group of heterogeneous tumors[2]. This heterogeneity contributes to significant differences in the responses of these tumors to the standard first-line treatment strategy, known as R-CHOP (rituximab, cyclophosphamide, doxorubicin hydrochloride, oncovin, and prednisone). Approximately 20%–50% of DLBCL patients experience relapse, or develop drug-resistance after treatment[1].

The current gold standard for lymphoma diagnosis is histopathologic examination of the affected tissue obtained through surgical resection or lymph node puncture, regardless of the expected NHL subtype. However, the World Health Organization (WHO) guidelines call for additional tests, such as flow cytometry, and cytogenetic tests like karyotyping and FISH, as they are essential for a more precise categorization and prognostication of DLBCL cases[3].

Literature Review:

For the diagnosis of lymphoma, international guidelines stipulate the need for a surgical open biopsy of the tissue involved in the suspected malignancy. The biopsied tissue is then sent to the pathology laboratory where a section is fixed for the evaluation of its structure and histomorphology, while the remaining sections are used to determine tumor clonality by flow cytometry, and for cytogenetic and molecular testing[4].

In several subtypes of NHL, especially DLBCL and the newly coined high-grade B-cell lymphoma (HGBL), precise categorization through cytogenetic testing -such as karyotyping and FISH- is crucial[5]. HGBL is a new subcategory of DLBCL based on the chromosomal abnormalities present in the malignant tissue. HGBL was previously known as double hit/triple hit DLBCL, where at least two specific cytogenetic abnormalities are present; one rearrangement involving the c-MYC oncogene, in addition to another rearrangement involving either BCL2 or BCL6 or both. The combination of the two (or three) translocations conveys an increased aptness for proliferation and apoptosis inhibition. Thus, the malignancy becomes more aggressive and refractory to conventional treatments[6].

Cytogenetic testing necessary for the detection of the aforementioned abnormalities is costly, time-consuming, and may not be readily available in routine laboratories. Moreover, karyotyping requires a fresh tissue sample which may be difficult to obtain[7]. Several prognostic indices are used internationally that do not take cytogenetic results into account. They rely on simple clinical parameters (*e.g.* patient age and tumor stage) while not taking into consideration neither the WHO recommended testing nor other potential prognostic risk factors currently being studied[8].

As a possible alternative for cytogenetic testing, recent studies have found that tissue oncogenic protein expression could be of prognostic significance in DLBCL[9].

c-MYC is a potent transcription factor involved in many cellular processes including proliferation. c-MYC protein overexpression may occur solely in B-cell malignancies without apparent gene aberrations, conferring a more aggressive tumor behavior[10].

Tumors expressing both the c-MYC and BCL2 proteins, i.e. double expressor (DE), also triple expressors (TE) expressing BCL6 in addition to c-MYC and BCL2, are all more aggressive and refractory to treatment similar to HGBL[7, 9, 11-13]. The effect of DE/TE status -regardless of chromosomal abnormalities- is being investigated as an independent prognostic parameter, hence, new treatment options are being sought[14]. Studies have also found prognostic implications for the overexpression of other proteins on their own, including BCL6[15].

Several other factors are implicated in DLBCL progression and prognosis such as subtypes based on

cell-of-origin (COO), whether it is activated B-cell (ABC) or germinal center B-cell (GCB). These subtypes are still not completely defined in terms of immunohistochemical or genetic characteristics. Current COO detection protocols use algorithms based on immunohistochemistry (IHC) tests not routinely implemented, namely BCL6 and MUM1[16].

In Syria, diagnosing lymphoma patients is achieved by histomorphologic testing and routine IHC alone, without applying the WHO required cytogenetic tests on account of their unavailability. No other prognostic factors or indices are being utilized. This makes the current protocol insufficient for the precise categorization of DLBCL and HGBL cases and may lead to suboptimal management.

Hence, our study aims to investigate auxiliary immunohistochemical tests not routinely used, namely c-MYC, BCL2 and BCL6, seeking a better prognostic testing protocol for DLBCL and HGBL patients.

Materials and Methods:

This research was designed as a retrospective observational study and has gained approval from the ethics committee at Damascus University.

Patient samples were collected retrospectively form the pathology laboratory at Al-Assad University Hospital in Damascus, Syria, and patient records were reviewed to obtain patient information and clinical data. Lab work was carried out at Al-Assad University Hospital laboratories.

Formalin-fixed paraffin-embedded (FFPE) tissue samples were included if the sample was obtained from the patient at diagnosis, and was indicative of DLBCL by histomorphologic examination and routine IHC tests.

Any sample that did not meet the above requirements was excluded.

Patient information and clinical data was followed up for a at least one year after diagnosis.

Forty-one samples meeting the inclusion criteria were collected from archived FFPE samples between 2014-2023. The 41 samples' clinical data was retrieved from the patients' medical records.

Four frosted slides were microtomed from each chosen FFPE sample with a thickness of 4-7mm. One of the four slides was stained with eosin-hematoxylin and re-examined by a pathologist to corroborate the malignancy and histomorphologic diagnosis in the chosen tissue sample.

The other three slides were used to apply IHC tests of the c-MYC, BCL2 and BCL6 oncoproteins. IHC was performed using rabbit monoclonal antibodies targeting each of the c-MYC, BCL2 and BCL6 proteins; clones 9E10, EP36 and EP278, respectively (Bio SB, USA). The tests were conducted using ImmunoDetector DAB HRP Brown kit (Bio SB, USA) according to manufacturer instructions. Positive IHC results were defined as ≥30% c-MYC expression, and ≥50% expression for BCL2 and BCL6.

Fisher's exact test was used to study the independence of protein IHC results in relation to one another and compared with data taken from patient records; progression-free survival (PFS) for one year after diagnosis, PFS for three- and five-years after diagnosis -if available-, bone marrow (BM) involvement, and extra-nodal presentation at diagnosis. Risk ratio (RR), odds ratio (OR), and their confidence intervals (CI) were also calculated, and Kaplan-Meier survival curves were graphed. GraphPad Prism 10 software was used for the statistical analysis. A **P** value of < 0.05 was considered statistically significant.

Results:

Immunohistochemical positivity for the c-MYC protein was seen in (18/41, 44%) patient samples. Statistical analysis of the c-MYC expression status (positive or negative) showed no correlation (P=0.72) neither with PFS for one year after diagnosis, nor with any of the other parameters studied.

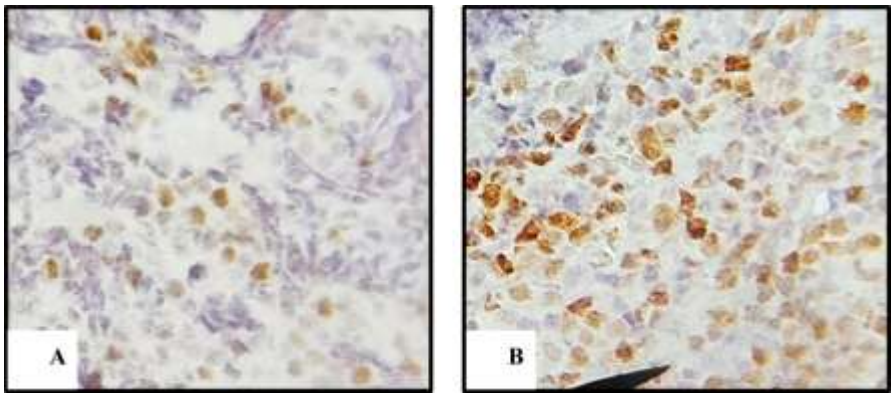

Figure (1): Positive IHC Results
A: c-MYC, B: BCL2.
Photomicrographs were taken with 40× objectives at 400× magnification

In addition, IHC positivity for the BCL2 protein was seen in (32/41, 78%) patient samples, which also did not implicate significance (P>0.05) in relation to PFS or any of the other parameters.

When considering c-MYC and BCL2 IHC positivity together (double expressor) (15/41, 37%) patient samples had DE status. There was no statistically significant association (P>0.05) between DE status and PFS or with most of the patient parameters. The only correlation seen (P=0.035) was between DE status and bone marrow involvement. [Figure 1] demonstrates microscope images of positive IHC results.

Whereas, when BCL6 IHC results were analyzed, BCL6 positivity showed a significant association (P<0.05) with unfavorable PFS outcomes - progression, relapse or mortality- within the first 3 years after diagnosis. Wherein, BCL6 positive patients had at least 10 times more of an unfavorable outcome during the first year than patients with negative BCL6 results (RR=10.42, OR=18.9, P=0.0025). Also, BCL6 positive patients were three times more likely to progress to an unfavorable outcome in comparison to BCL6 negative patients within three years of diagnosis (RR=3.125, OR=6.667, P=0.0159). Our findings indicated no further correlation between BCL6 positivity and PFS up to 5 years, or the other parameters. Albeit, BCL6 positivity had weak statistical significance in relation to poor overall survival (P=0.057). [Table 1] lists those correlations in more detail.

Table (1): Correlation between BCL6 IHC results and patient parameters

Parameter		N	BCL6 (+)	BCL6 (-)	RR	RR CI	OR	OR CI	P
PFS 1Y	No*	41	9 (22%)	1 (3%)	10.42	1.987 to 61.01	18.9	2.696 to 215.0	0.0025
	Yes		10 (24%)	21 (51%)					
PFS 3Y	No*	36	10 (28%)	4 (11%)	3.125	1.297 to 8.201	6.667	1.433 to 23.89	0.0159
	Yes		6 (17%)	16 (44%)					
PFS 5Y	No*	32	8 (25%)	5 (16%)	2.057	0.8890 to 4.989	3.467	0.7940 to 14.26	0.1492
	Yes		6 (19%)	13 (41%)					
Overall Survival	No†	41	7 (17%)	2 (5%)	4.053	1.106 to 15.96	5.833	1.202 to 30.01	*0.057*
	Yes		12 (29%)	20 (49%)					
BM Involvement	Yes	41	6 (15%)	2 (5%)	3.474	0.9162 to 14.00	4.615	0.8764 to 24.26	0.1152
	No		13 (32%)	20 (49%)					
Extra-Nodal Presentation	Yes	41	10 (24%)	15 (37%)	0.772	0.4406 to 1.271	0.519	0.1325 to 1.788	0.3522
	No		9 (22%)	7 (17%)					

*: progression, relapse, or mortality. †: mortality
PFS: progression-free survival, BM: bone marrow, RR: risk ratio, OR: odds ratio, CI: confidence interval

Triple expressor status (TE; c-MYC, BCL2, and BCL6 all positive) was seen in only eight patients (8/41, 20%), but was significantly correlated with several parameters, including poor outcomes for one year and 3-year PFS (RR=4, RR=3, respectively, P<0.05). An association was also found between TE with BM involvement and extra-nodal presentation (P<0.05). Mortality due to disease progression was four times more likely in TE patients, though statistical significance was weak (P=0.0544). [Table 2] lists all TE correlations in detail.

Table (2): Correlation between triple expressor status and patient parameters

Parameter		N	TE	Non-TE	RR	RR CI	OR	OR CI	P
PFS 1Y	No*	41	5 (12%)	5 (12%)	4.125	1.514 to 10.44	9.333	1.474 to 41.19	0.0129
	Yes		3 (8%)	28 (68%)					
PFS 3Y	No*	36	6 (17%)	8 (22%)	3.107	1.474 to 6.067	15.75	1.737 to 187.2	0.0083
	Yes		1 (3%)	21 (58%)					
PFS 5Y	No*	32	4 (13%)	9 (28%)	2.4	0.9957 to 4.616	8	0.9619 to 102.2	0.1316
	Yes		1 (3%)	18 (56%)					
Overall Survival	No†	41	4 (10%)	5 (12%)	3.3	1.100 to 8.899	5.6	1.226 to 26.64	*0.0544*
	Yes		4 (10%)	28 (68%)					
BM Involvement	Yes	41	4 (10%)	4 (10%)	4.125	1.292 to 12.12	7.25	1.484 to 41.14	0.0333
	No		4 (10%)	29 (70%)					
Extra-Nodal Presentation	Yes	41	8 (20%)	17 (41%)	1.941	1.238 to 2.994	N/A	N/A	0.0144
	No		0 (0%)	16 (39%)					

*: progression, relapse, or mortality. †: mortality
PFS: progression-free survival, BM: bone marrow, RR: risk ratio, OR: odds ratio, CI: confidence interval

[Figure 2] demonstrates Kaplan-Meier curves displaying the probability of survival for patients inversely correlated with positive BCL6 IHC and TE (p<0.05), further highlighting the added prognostic significance of these parameters.

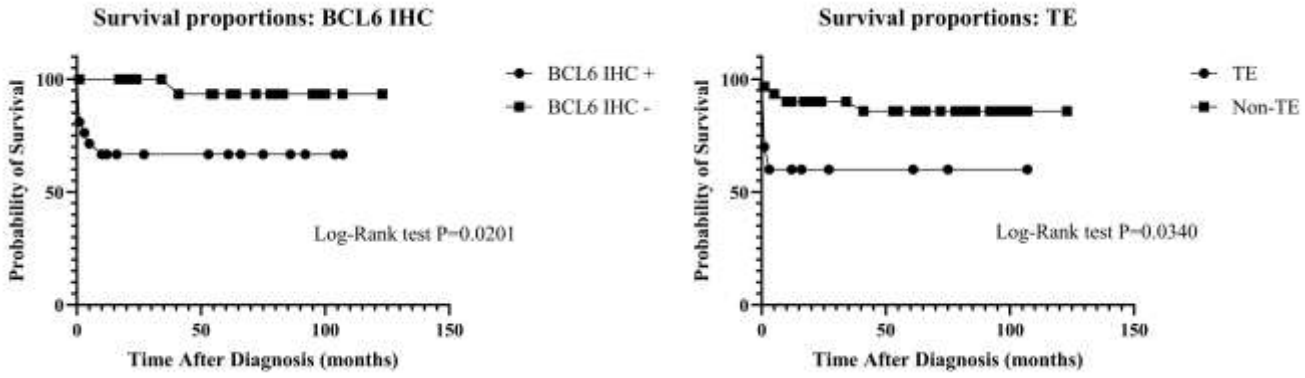

Figure (2): Kaplan-Meier Curves For BCL6 IHC and TE Status

Kaplan-Meier curves displaying statistically significant effects on probability of patient survival for BCL6 IHC results (Log-Rank test P=0.0201) and TE status (Log-Rank test P=0.034)

Discussion:

Our study showed that the overexpression of c-MYC alone did not convey a worse prognosis for the patient in any regard. As c-MYC is involved in many cellular processes, including cell cycle transition, in addition to promoting apoptosis, a tumor with the overexpression of c-MYC may have increased proliferation, but with it a higher rate of apoptosis, which would limit tumor growth. Thus, c-MYC deregulation alone is neither the main tumorigenic factor nor the sole cause of the aggressive clinical behavior in DLBCL[17].

Also, our findings implicated no statistically significant correlation between double expressor status and disease progression or overall survival. Even though, most previous studies suggested prognostic implications for DE status[9, 18-20], some other studies did not find a strong predictive value for DE in DLBCL patients[21-23]. Albeit, the sample size we were able to analyze is small, our findings provided evidence against a predictive value of DE status. Thus, the routine IHC detection of c-MYC and BCL2 proteins concurrently is not recommended.

Our results showed that either BCL6 IHC positivity alone or triple expressor status were similarly correlated with unfavorable patient outcomes and poor overall survival. In agreement with the results in our study, other studies have identified BCL6 as a potentially poor prognostic factor in DLBCL[15, 24, 25]. The significance of BCL6 overexpression may be a reflection of BCL6 being linked to activated B-cell (ABC) subtype, which, in turn, is a more aggressive form of DLBCL[16, 25]. In contrast to our study, some previous studies have found favorable prognostic value for BCL6 overexpression in DLBCL patients[26, 27].

As evidenced by our results; higher risk- and odds-ratios for BCL6 positivity on its own -as it is linked to unfavorable progression of disease- the detection of TE status, which is a more costly approach, did not add significance to the prognosis of disease progression or survival in DLBCL patients. Accordingly, the detection of BCL6 protein overexpression alone is adequate, and can be suggested as a promising routine immunohistochemical test for enhancing the inference of prognosis in DLBCL.

Conclusions:

Although our study is limited by the small number of samples we were able to analyze, we can conclude that for the determination of DLBCL prognosis, routine IHC testing for BCL6 protein overexpression is recommended.

We suggest that BCL6 IHC testing should be added to the workup of DLBCL patients in Syria.

Abbreviations:

BM	Bone marrow
CI	Confidence interval
DE	Double expressor
DLBCL	Diffuse Large B-Cell Lymphoma
IHC	Immunohistochemistry
NHL	Non-Hodgkin Lymphoma
OR	Odds ratio
OS	Overall survival
PFS	Progression-free survival
RR	Risk ratio
TE	Triple expressor

التمويل : هذا البحث ممول من جامعة دمشق وفق رقم التمويل (501100020595).

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