Journal of Molecular Biomarkers & Diagnosis

Rojas, J Mol Biomarkers Diagn 2016, 7:2
DOI: 10.4172/2155-9929.7: 276

Research Article Open Access

Oct2, BCL6, IRF8, OCAB and PU.1 in the Assessment of Prognosis in Diffuse Large B cell Lymphoma Patients

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Rec Date: Jan 20, 2016, **Acc Date:** Feb 06, 2016, **Pub Date:** Feb 06, 2016

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Abstract

Background: Diffuse Large B-Cell Lymphoma (DLBCL) is the most common type of Non-Hodgkin Lymphoma in adults. This germinal center derived B cell lymphoma is a heterogeneous disease with a highly variable clinical course, currently treated with immune-chemotherapy. The International Prognosis Index (IPI) remains the main prognosis indicator. This highlights the absence of biomarkers suitable to provide molecular biology information to more accurately establish prognosis and predict treatment response in DLBCL patients.

Methods: We determined the Oct2, BCL6, IRF8, OCAB and PU.1 transcription factors expression by immunohistochemistry in 73 DLBCL lymph node biopsies to address their potential as prognosis biomarkers in DLBCL patients. These molecules exhibit well-known key roles in the germinal center development.

Results: A large number of cases showed high Oct2 (64/73), BCL6 (40/73) and/or IRF8 (44/73) percentage of positive tumor cell nuclei. In contrast, a significant number of analyzed biopsies, showed a low OCAB and/or PU.1 percentage of positive tumor cells. The expression of each factor was not associated with any of the relevant clinical-pathological features including the DLBCL molecular subtype and the IPI. Oct2, BCL6 and IRF8 high expression (more than 70% of positive tumor cells) correlated with poor prognosis in terms of shorter overall survival. Particularly, high BCL6 and IRF8 expression maintained their prognostic value in a multivariate analysis stratified for the IPI score.

Interestingly, IRF8 emerged as a novel prognosis indicator among the free bone marrow disease patients at diagnosis, subjected to a specific multivariate analysis named classification tree. Patients with free-bone marrow disease, which normally have a better outcome, showed a worse prognosis when they expressed high IRF8 at diagnosis.

Conclusions: The assessment of these factors expression would provide novel cellular and molecular insights to more efficiently predict DLBCL patient prognosis.

Keywords: DLBCL, Prognosis biomarker, Germinal center, Oct2, BCL6, IRF8, OCAB, PU.1

Abbreviations

GC: Germinal Center; TF: Transcription Factor; NHL: Non-Hodgkin Lymphoma; DLBCL: Diffuse Large B Cell Lymphoma; IPI: International Prognosis Index; LDH: Lactate Dehydrogenase; GCB-Like DLBCL: Germinal Center B-Cell-Like DLBCL; ABC-Like DLBCL: Activated B Cell-Like DLBCL; Md: Median; DFS: Disease Free Survival; OS: Overall Survival; IHC: Immunohistochemistry; R-CHOP: Rituximab; Cyclophosphamide; Hydroxydaunomycin; vincristine; prednisone

Introduction

Germinal centers (GCs) are a dynamic immune system compartment specialized in generating high-affinity antibody-producing cells. GCs form when T cell–dependent activation induces the migration of B cells to lymphoid follicles, where they differentiate into centroblasts and up-regulate the transcriptional repressor BCL6 and AID (activation-induced cytosine deaminase) [1,2].

Transition from a naïve B cell through the GC stages of B cell maturation and further differentiation is tightly controlled by a sequential activation and down-regulation of key transcription factors (TFs), facilitating in a high timely organized process the pre-GC, the GC and the post-GC B-cell program execution [3]. Among many other

TFs, Oct2, OCAB, BCL6, PU.1 and IRF8 play important roles during GC formation.

The octamer binding protein Oct2, which expression pattern is more limited to lymphoid cells as compared to the ubiquitously expressed Oct1, belongs to the POU-domain family of TFs [4]. Oct2 functions with the B-lymphocyte restricted co-activator named OCAB (Octamer Co-Activator from B cells) [5]. OCAB, also known as OBF-1 and BOB.1, interacts with the octamer DNA binding proteins, Oct1 and Oct2, and together with either of them mediates efficient cell type-specific transcription of immunoglobulin promoters [5-8]. Mice that lack Oct2 die at birth for suspected but undetermined reasons [9]. B-cells with mutated Oct2 do not proliferate normally in vitro and are blocked in the G1 phase of the cell cycle, suggesting a role in B cell proliferation [9]. Regarding its co-factor, mice that lack OCAB are viable, show unaffected B cell development in the bone marrow, have reduced B cell number in the spleen and fail to form GCs [10].

Crucial genes regulated by Oct2 and OCAB that might conclusively contribute to explain their role in normal GC B-cells remain largely elusive. It has been indicated that Oct2 and OCAB are highly expressed at the protein level in certain types of hematological malignancies compared to the normal cell counterpart but nonetheless there is very little information regarding the role of Oct2 or OCAB in malignant transformation [11]. It has been suggested that Oct1, Oct2 and OCAB mediate cell survival in t[8;14] follicular lymphoma cells by directly activating the anti-apoptotic gene bcl-2 therefore implicating these factors in the acquisition of a malignant phenotype [12].

Although many of the details regarding GC function and maturation of B cells remain controversial, it is known that up-regulation of BCL6 is required for entry of B cells into the centroblast [CB] phase as well as for maintenance of B cells in the GC compartment by blocking further differentiation [13-15]. BCL6 is a member of the 'BTB-POZ' (bric a´ brac, tramtrack, broad complex–poxvirus zinc finger) family of TFs and represses genes by recruiting several different co-factors complexes to the BCL6 BTB domain [16]. Interaction with these copartners is required for the 'CB-licensing' effect of BCL6, as a specific inhibitor of the BCL6 BTB domain (BPI), blocks GC formation in vivo after T cell–dependent antigen stimulation [17]. Furthermore, constitutive expression of BCL6 due to translocation or point mutations in the promoter elements of its gene is the most common genetic lesion in B cell lymphomas [18] and can induce diffuse large B cell lymphoma (DLBCL) in mice [19]. The 'GC–licensing' activity of BCL6 is tightly linked to its effects on lymphomagenesis; in addition to blocking the formation of GCs, BPI kills lymphoma cells that express BCL6 constitutively [19]. BCL6 target genes have been extensively studied and it is well known for repressing genes involved in cell cycle regulation and differentiation in normal B cells [20]. More recently it has been elucidated the BCL6 induced mechanism that allows the GC B cells high proliferation rate meanwhile undergoing extensive DNA rearrangements. BCL6 impairs CBs´ DNA damage sensing and consequently inhibits the cell death response [21-23].

The Ets (E-twenty six) family of TFs PU.1 and the IRF (Interferon Regulatory Factor) family of TFs IRF8, are highly expressed in GC B cells. Although IRF8 is detectable in most reactive B-cells, GC B cells contain the highest levels of IRF8, with lower levels seen in mantle and marginal zone B cells and none in plasma cells. IRF8 is most strongly expressed in lymphomas of GC origin with lower levels present in mantle cell lymphomas, chronic lymphocytic leukemia, marginal zone lymphomas, and no expression observed in plasmacytic/plasmablastic neoplasms [24]. PU.1 is a critical TF for both myeloid and lymphoid cells differentiation. PU.1 knockout mice die within forty eight hours after birth due to a severe septicemia [25,26]. Regarding the B cell lineage, PU.1 is expressed uniformly throughout the mature pre-plasma cell B cell population, the only exception being a subpopulation of GC cells which showed exceptionally high PU.1 levels. Expression of PU.1 and BCL6 was also found to be up-regulated in CB in the normal GC, but jointly down-regulated in a subpopulation of centrocytes [27].

DLBCL is the most common B cell Non Hodgkin Lymphoma [NHL] in adults [28]. In the United States, 15,000 new cases of DLBCL are diagnosed each year with over 50% of untreated patients surviving less than a year [29]. Applying the standard-of-care treatment [rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone], approximately 80% to 85% of patients achieve a complete remission, but a significant minority [20% to 25%] of these patients will relapse. Relapse or primary refractory DLBCL remain the major cause of treatment failure and death in this disease [30-32]. The International Prognosis Index (IPI) is a clinical tool developed by oncologists to aid in predicting the prognosis of patients with aggressive NHL. It assigns patients a score based on the simultaneously evaluation of age, serum LDH (Lactate Dehydrogenase) level, tumor stage, performance status and the disease extra-nodal involvement at the diagnosis [33]. This clinical-pathological parameter lacks the information that the cellular and molecular biology insights can provide to more effectively assess DLBCL patient risk at diagnosis and predict response to therapy [34-37].

Gene expression profiling has classified DLBCL into two subgroups named as Activated B Cell-Like [ABC] and Germinal Center B Cell-Like [GCB] [38]. Multiple mutations associated with their biology have been identified by next generation sequencing but little is known about their prognostic role [39-41]. We performed the tissue expression analysis of Oct2, BCL6, IRF8, OCAB and PU.1 in DLBCL lymph node biopsies. Their potential use as prognosis biomarkers was assessed.

Materials and Methods

Patient Samples

For this retrospective study paraffin embedded DLBCL lymph node biopsies of 73 untreated patients [38 male median age and range 59 [25-85]; 35 female 59.4 [17-82]] diagnosed during the 2004-2014 period were analyzed. Patient samples were obtained from the Pathology Department of the Institute of Oncology "Ángel H. Roffo".

All relevant clinical and histopathological data, as well as information on therapies applied were collected from the clinical charts. We included sex, age, DLBCL stage and molecular subtype, serum LDH levels at diagnosis, lymphatic nodes involved, bone marrow infiltration, extra-nodal disease at diagnosis, B symptoms and the IPI. Table 1 summarizes the main studied population characteristics.

The follow-up period lasted a median of 39 months with a range of 6–190 months. All patients who died had clear evidence of uncontrolled tumor growth at the time of death. Disease Free Survival (DFS) was considered the length of time between absence of disease determined by imaging and the reappearance of signs or symptoms confirmed by the same imaging methods. Overall survival (OS) was determined as the time length after the diagnosis up to the patient death or last recorded information.

This protocol was reviewed and approved by the Ethical Committee of the Institute of Oncology.

Feature		N of patients
Sex	Male	38
	Female	35
Age group (years)	20-30	5
	31-40	2
	41-50	7
	51-60	22
	61-70	19
	>70	18
Lymphoma Stage	I	18
	II	26
	III	15
	IV	14
Lymphadenopathy Topography	Cervical	28
	Mediastinum	1
	Axillary	5
	Inguinal	9
	Retroperitoneal	3
	Multiple	23
B Symptoms	Absent	50
	Present	23
International Prognosis Index	1	34
	2	19
	3	14
	4	6
		Median (range)
Follow-up period		39 (6-190) months
Events during the follow-up		
Relapsed Patients		35
Dead Patients		45
Note: When columns do not sum to the total, data were missing or unknown.		

Table 1: Some of the main clinical and pathological features of the studied population are summarized in this table. The median length of the follow-up period and the patient outcome are shown.

Immunohistochemistry (IHC)

Oct2, BCL6, IRF8, OCAB, PU.1 and the corresponding normal mouse or rabbit serum control stains were performed on formalin-fixed, paraffin-embedded sections of lymph node biopsies from DLBCL patients.

All the primary antibodies αOct2 [PT2] sc-56822, αBCL6 [N-3] sc-858, αIRF8 [E-9] sc-365042, αOCAB [C-20], αPU.1 [Sp1-1] [T-21] sc-352, normal mouse sera sc-45051 and normal rabbit sera sc-2338 were purchased from Santa Cruz Biotechnology, Inc [Dallas, Texas USA].

Representative serial sections [5- μm thick] were placed on positively charged slides. Sections were deparaffinized and rehydrated in graded alcohol and then placed in a low pH antigen retrieval solution [10 mM Citrated Buffer pH 6] and steamed for 20 minutes to recover antigenicity. Activity of endogenous peroxidase was achieved by incubation with 3% H_2O_2 at room temperature for five minutes. Sections were blocked with dried skim milk for 60 minutes at room temperature, followed by the first antibody, incubated overnight at 4°C. The universal biotinilated secondary antibody was applied during 1 hour at room temperature ([provided by the Vectastain ABC kit Universal, Vector Laboratories, Burlingame, CA). After washing, sections were treated with Vectastain ABC kit Universal [Vector Laboratories, Burlingame, CA] for 1 hour at RT, following manufacturer´s instructions and then incubated with the chromogen 3, 3'-diaminobencidine [7%] plus 3% H_2O_2 in water [DAB Peroxidase Substrate, SK-4100]. Finally, they were counterstained with Harris hematoxylin. Negative controls, replacing the primary antibody for the corresponding rabbit or mouse normal serum, were performed to discriminate background staining.

The expression of the different antigens was analyzed by three independent observers and scored according to the number of positive cells. Positivity was defined as nuclear bright brownish staining of the neoplastic cells nuclei. Differences in the intensity of staining were not considered for the analysis. The occasional cytoplasmic staining seen with some antibodies was considered as nonspecific. The labeling index (LI) for each antibody was calculated as the percentage of labeled malignant cells out of the total number of tumor cells counted.

Statistical Analysis

Bivariate relationships between the studied variables and various clinical-pathological parameters were evaluated statistically using the Chi Square Test. Correlation between variables was assessed by Pearson correlation coefficients. A difference of $p < 0.05$ was considered to be significant. We used linear regression to summarize the joint effects of the IPI score and the antigen positivity. The Kaplan–Meier method was used to estimate overall survival. In univariate survival analyses, two-sided log-rank test for equality of survivor functions were used to assess the prognostic significance of different parameters on Oct2, BCL6, IRF8, OCAB and PU.1 positivity. Multivariate analysis was performed using the stepwise Cox proportional hazards model to evaluate the predictive power of each variable independently of the others. We employed a method starting with the Cox model containing all four variables and successively eliminated the least statistically significant variable until only significant variables were left [$p < 0.05$]. All variables were entered in the multivariate analysis as categorical ones. SPSSPC+ [version 10] for Windows software was used for the aforementioned analyses.

Finally, we performed an additional multivariate analysis by creating a decision or classification tree to assess the effect of specific variables on survival, including known predictors of DLBCL prognosis [stage,

age, serum LDH levels, etc.] and the variables Oct2, BCL6 and IRF8 expression which were found to be significant by univariate analysis.

This model is designed to capture additive behaviors as standard linear models do not allow interactions between variables unless they specify a multiplicative form. This method is called classification tree as the original method of presenting it is as a binary tree.

The method consists in the data set partitioning. Initially all objects are considered as belonging to the same group. The group is split into two subgroups from one of the regressive variables so that the heterogeneity at the level of the dependent variable is minimal. The two subgroups [nodes] formed are separated again if sufficient heterogeneity to produce a partition observation and / or if the size of the node is greater than the minimum established to continue the algorithm. The process ends when any of these conditions is met.

Results

Oct2, BCL6, IRF8, OCAB and PU.1 expression analysis by IHC

We evaluated the Oct2, BCL6, IRF8, OCAB and PU.1 expression in 73 lymph node biopsies of untreated DLBCL patients.

Oct2, BCL6 and IRF8 TFs showed high expression in patient samples. We considered "high Oct2, BCL6 or IRF8 expression" when the biopsies showed more than 70% of stained tumor cell nuclei. In this regard 64/73 [87.7%], 40/73 [54.8%] and 44/73 [60.3%] biopsy samples were "high" for Oct2, BCL6 and IRF8 expression respectively (Figure 1). Strikingly, both OCAB and PU.1, also highly express in B cells during their normal transit through the GC, showed predominantly low expression levels in DLBCL lymph node biopsies (Figure 1). Thus, applying the same cut-off point [of > 70%], only 18/73 [24.6%] and 11/73 [15.1%] of the biopsies were high for the co-activator OCAB and the Ets TF PU.1 respectively. Furthermore, OCAB and PU.1 expression was completely lost in 42.5% and 63% of patients. According to the level of expression observed for these two molecules, a cut-off point value of more than 10% of stained tumor cell nuclei, was applied to define OCAB and PU.1 expression as "high" in the cohort of patients evaluated. Using this cut-off value of > 10%, 39/73 [53.4%] samples were high for OCAB and 18/73 [24.6%] were high for PU.1.

Association of Oct2, BCL6, IRF8, OCAB and PU.1 tissue expression with the clinical and pathological parameters

There was no association between the percentage of Oct2, BCL6, IRF8, OCAB and PU.1 positive tumor cells individually analyzed and the clinical-pathological parameters including sex, age, lymphoma stage, B symptoms, bone marrow infiltration, extra-nodal disease, DLBCL molecular subtype and serum level of LDH at diagnosis (Chi Square Test, p = NS) (Table 2).

To summarize the lack of correlation between the antigen immunostaining and current relevant clinical-pathological features, the IPI results are shown in Table 3, which assigns patients a risk score at the diagnosis of the disease, analyzing simultaneously several of the main currently relevant prognosis indicators (age, serum LDH, functional status, number of extra-nodal sites of disease and lymphoma stage).

Correlations among Oct2, BCL6, IRF8, OCAB and PU.1 expression levels

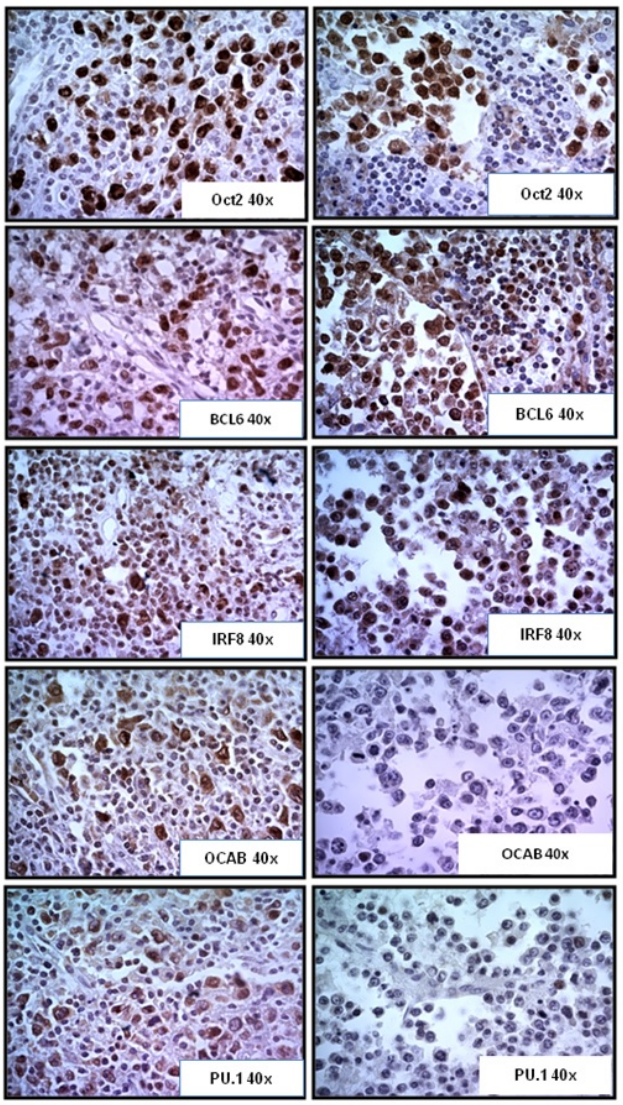

Figure 1: Expression of Oct2, BCL6, IRF8, OCAB and PU.1 in DLBCL lymph node biopsies by immunohistochemistry. A DLBCL patient biopsy with high Oct2, BCL6, IRF8, OCAB and PU.1 expression in lymphoma cells nuclei (left). A DLBCL patient biopsy with high Oct2, BCL6 and IRF8 and low OCAB and PU.1 expression (right).

Similarly there was a lack of correlation both with the immune-phenotype markers routinely evaluated [CD3, CD5, CD10, CD15, CD20, CD23, CD30, CD45, CD68, CD138 or BCL2] and the growth fraction marker Ki67, despite that the analyzed molecules are involved in GC B cell proliferation control [Data not shown].

In a multiple stepwise linear regression analysis, where the BCL6 or IRF8 percentage of positive tumor cells were the dependent variables, they remained independent of the IPI score (p = 0.03 and p = 0.04 respectively).

The percentage of Oct2 expression correlated with BCL6 (Pearson correlation test $p < 0.05$) and IRF8 [Pearson correlation test $p < 0.01$] expression, as well as PU.1 correlated with OCAB (Pearson correlation test $p < 0.01$) and IRF8 (Pearson correlation test $p < 0.01$) expression levels.

The potential correlations among the five molecules expression levels were analyzed considering all the possible combinations (both factors high, one of them high and the other one low and both of them low).

Survival analysis of DLBCL patients

Disease Free Survival (DFS)

Oct2, BCL6, IRF8, OCAB or PU.1 did not predict DFS in this cohort of 73 DLBCL patients using the corresponding cut off point defined for each transcription factor.

Parameter	Oct2 + / Total (%)	BCL6 + / Total (%)	IRF8 + / Total (%)	OCAB + / Total (%)	PU.1 + / Total (%)
Sex					
Male	34/39 (87.2)	22/39 (56.4)	22/39 (56.4)	18/39 (46.1)	11/39 (28.2)
Female	30/34 (88.2)	18/34 (52.9)	22/34 (64.7)	21/34 (61.7)	7/34 (20.6)
Age					
≤ 60	31/36 (86.1)	20/36 (55.5)	21/36 (58.3)	21/36 (58.3)	8/36 (22.2)
> 60	33/37 (89.2)	20/37 (54.0)	23/37 (62.2)	18/37 (48.6)	10/37 (27.0)
DLBCL Stage					
I	14/17 (82.4)	11/17 (64.7)	14/17 (82.4)	14/17 (82.4)	14/17 (82.4)
II	23/26 (88.5)	14/26 (53.8)	23/26 (88.5)	23/26 (88.5)	23/26 (88.5)
III	13/15 (86.7)	6/15 (40.0)	13/15 (86.7)	13/15 (86.7)	13/15 (86.7)
IV	13/14 (92.9)	8/14 (57.1)	13/14 (92.9)	13/14 (92.9)	13/14 (92.9)
LDH serum levels					
Normal	13/15 (86.7)	8/15 (53.3)	10/15 (66.7)	6/15 (40.0)	2/15 (13.3)
Up	20/21 (95.2)	10/11 (90.9)	14/21 (66.7)	12/21 (57.1)	4/21 (19.0)
Extranodal disease					
Absent	22/27 (81.5)	14/27 (51.8)	15/27 (55.5)	12/27 (44.4)	2/27 (7.4)
Present	27/29 (93.1)	15/29 (51.7)	18/29 (62.0)	20/29 (69.0)	9/29 (31.0)
B symptoms					
Absent	42/49 (85.7)	31/49 (63.3)	27/49 (55.1)	27/49 (55.1)	15/49 (30.6)
Present	21/23 (91.3)	8/23 (34.8)	16/23 (69.6)	11/23 (47.8)	3/23 (13.0)
Pearson Chi Square Test: NS					

Table 2: No association was found between Oct2, BCL6, IRF8, OCAB or PU.1 expression and the clinical and pathological parameters currently considered as relevant in DLBCL patients. The table shows the lack of correlation with sex, age, DLBCL stage, serum LDH level, extranodal disease and B symptoms, as examples. The entire set of data (clinical, pathological, phenotype markers and treatments) was collected from patient charts and analyzed in the same way applying the Chi Square Test. Other parameters included were DLBCL molecular phenotype (GCB and non-GCB), topography of lymph nodes involved, treatments received and patient response to therapy.

Nevertheless, PU.1 expression predicted a shorter DFS using the Kaplan-Meier method [Long Rank Test X2, $p = 0.003$] when analyzed using the 70% cut off value. In fact among the relapsed group of patients, 77% off them showed low PU.1 expression levels (less than 70% of stained tumor cell nuclei) and 50% of them relapsed during the first 20 months following the initial diagnosis.

Overall Survival (OS)

The Kaplan-Meier study showed that tumors with Oct2 [[Log Rank Test X2 = 3,7] $p = 0.04$], BCL6 [[Log Rank Test X2 < 4,0]; $p = 0.04$] or IRF8 expression [[Log Rank Test X2 4,0]; $p = 0.04$] higher than 70% correlated with poor prognosis in terms of reduced overall survival (Figure 2).

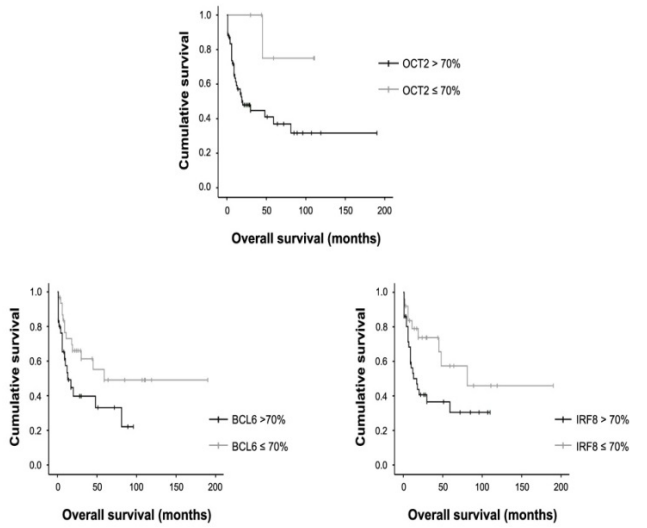

Figure 2: *The Oct2, BCL6 and IRF8 expression correlated with DLBCL overall survival.* The Kaplan-Meir overall survival curves show that patients with a high percentage of Oct2 (A), BCL6 (B) or IRF8 (C) stained tumor cell nuclei (more than 70%) had a poor outcome in terms of shorter overall survival.

In the study stratified for IPI, Cox-proportional Hazards analysis, showed that high percentage of BCL6 ($p < 0.03$) and IRF8 ($p < 0.03$) positive tumor cells, still predicts overall survival. However, the multivariate analysis disclosed that the Oct2 correlation with OS lost significance when the co-variable IPI was included in the model (Data not shown).

Parameter	Oct2 + / Total (%)	BCL6 + / Total (%)	IRF8 + / Total (%)	OCAB + / Total (%)	PU.1 + / Total (%)
IPI					
1	27/33 (81.8)	20/33 (60.6)	21/33 (63.6)	14/33 (42.4)	7/33 (21.2)
2	18/19 (94.7)	11/19 (57.9	13/19 (68.4)	9/19 (47.4)	4/19 (21.0)
3	13/14 (92.8)	5/14 (35.7)	7/14 (50.0)	10/14 (71.4)	4/14 (28.6)
4	5/6 (83.3)	3/6 (50.0)	2/6 (33.3)	5/6 (83.3)	3/6 (50.0)
Pearson Chi Square Test: NS					

Table 3: No association was found between the expression of Oct2, BCL6, IRF8, OCAB or PU.1 and the International Prognosis Index score, currently considered as the main prognosis indicator in these patients.

The combined analysis of BCL6 and IRF8 expression levels showed that patients who presented high BCL6 and IRF8 or high BCL6 and low IRF8 at diagnosis had a shorter overall survival as compared to patients that had low level expression of both factors [[Log Rank Test X2 = 7,0] p = 0.03] (Figure 3).

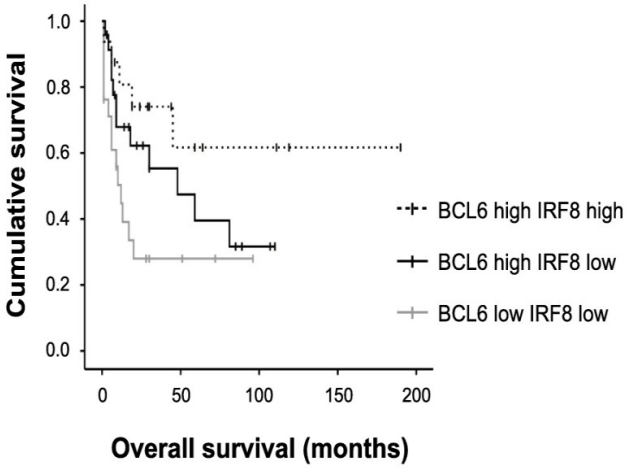

Figure 3: *Overall survival Kaplan-Meir curve for combined expression of BCL6 and IRF8.* Patients who showed low expression levels of both BCL6 and IRF8 at diagnosis had a better outcome with a longer overall survival.

When the cut-off point value established to define positive PU.1 or OCA-B expression was employed, these two TFs did not predict OS. Despite this, if we analyzed PU.1 expression levels applying the 70% cut-off point, we found that low PU.1 expression levels or its loss correlated with shorter OS [p = 0.02]. Among patients who died, 74.2% of them showed low PU.1 expression at diagnosis. Furthermore, 46/73 patients had no PU.1 expression

Decision or classification trees

We used a tree-structured model to evaluate OS in a group of 36 patients of which the bone marrow infiltration data at the diagnosis of the disease was available. The bone marrow infiltration status, the IPI score, BCL6, IRF8 and Oct2 expression, were considered as the regressive variables.

Variable	OS (n = 36)						
First Node	No bone marrow infiltration (n = 31)				Bone marrow infiltration (n = 5)		
Second Node	IRF8 low (n = 16)		IRF8 high (n = 15)		Stage ≤ 3 (n = 3)	Stage = 4 (n = 2)	
Third Node	ITI = 1 (n = 7)	ITI ≥ 2 (n = 9)	ITI = 1 (n = 3)	ITI ≥ 2 (n = 12)	----------	Male (n = 1)	Female (n = 1)

Table 4: The IRF8 expression at diagnosis provides valuable information to discriminate among the clinically considered low risk

DLBCL patients. IRF8 expression predicts outcome among this group of DLBCL patients in terms of overall survival.

In this cohort of patients, bone marrow infiltration was the main feature to predict OS, so it was allocated to node I. In this regard, patients that showed bone marrow infiltration at diagnosis showed 3.3 times higher risk of a poor outcome in terms of OS [Log Rank Test X2 = 7,0 p = 0.006].

Patients with a better clinical prognosis [without bone marrow infiltration] were further dichotomized by the percentage of IRF8 positive tumor cells being in the second node. Patients free of bone marrow disease but that showed high IRF8 expression levels at diagnosis had a shorter OS. So IRF8 expression levels proved to be a better indicator of prognosis before the IPI in the subgroup of patients with no bone marrow infiltration (Table 4)

Discussion

NHL is the most common cancer of the lymphatic system being DLBCL the most prevalent type [42]. Even though in recent years considerable progress has been made in the treatment of patients with B-cell NHLs and although responses can be achieved with combined chemo-immunotherapy regimens, patients with indolent lymphomas continue to relapse and at least a third of DLBCL cases would never be cured with current therapies. It was only with the addition of the monoclonal antibody against the pan-B-cell antigen CD20 to the conventional chemotherapy scheme that an improvement in the outcome of patients was achieved especially in the low-risk group according to the IPI score in terms of overall four-years and same period progression free survival [43-50]. It is now clear that one of the obstacles to therapeutic success in DLBCL is the heterogeneous nature of this disease, which can be appreciated at the morphologic, genetic, phenotypic, and clinical level.

We analyzed the Oct2, BCL6, IRF8, OCAB and PU.1 expression in 73 lymph node biopsies of DLBCL patients. These TFs play crucial roles in B-cell development and differentiation. They are both expressed in normal GC B-cells and in GC cells within reactive lymphoid hyperplasia [51].

The DLBCL patients cohort evaluated herein showed a large percentage of high Oct2 [87.7%], BCL6 [54.8%] and IRF8 [60.3%] expressing cases. The opposite was observed for OCAB and PU.1 expression. No association was found between these TFs individually evaluated and the clinical and pathological features currently taken into account to assess DLBCL patient prognosis. We highlight the lack of association between these molecules and the IPI risk score, since this parameter is universally used by clinical oncologists as the main prognosis factor for this disease.

We found that when more than 70% of neoplastic cells showed Oct2, BCL6 or IRF8 nuclear expression, patients had worse outcome and reduced OS. Interestingly, this association remained significant when the multivariate analysis was stratified according to the IPI score for BCL6 and IRF8. Even though further studies are necessary, specially including a larger number of patients, our finding implies that the expression level of both BCL6 and IRF8 at diagnosis could be useful to predict DLBCL patient prognosis independently of their age, status performance, lymphoma stage, LDH serum levels and extra-nodal sites of disease at diagnosis. Furthermore, IRF8 expression was able to predict OS in patients without bone marrow infiltration at diagnosis even better than the currently used IPI.

There have been various approaches to differentiate between separate nosological entities within DLBCL, based on various methods, such as the microarray or IHC techniques. Although it has been proven that gene expression profiling using cDNA microarrays could identify prognostically important DLBCL subgroups, this method is impractical as a clinical tool. Therefore, investigators have started using immune-staining methods in their studies. Since the IHC tissue microarray reported by Hans, different algorithms have been proposed to classify DLBCL into molecular subtypes, based on the expression of several markers [52]. Hans sub-classified DLBCL cases using CD10, BCL6, and MUM1 expression into GCB or non-GCB [52]. Subsequent studies yielded conflicting results regarding this algorithm prognostic value. The controversies could be the consequence of the availability of new antibodies raised against GC markers and treatment received by patients included in the different studies performed, especially considering patients treated before or after the R-CHOP era [53-56]. Later on, the Hans algorithm was improved studying a group of CHOP-treated DLBCL patients using GCET1, CD10, BCL6, MUM1 and FOXP1 immunostains [56]. Although the Nyman and the Muris algorithms showed a high concordance with the microarray results, each has a low value for either sensitivity or specificity [54, 57]. As mentioned before the most important clinical predictor of survival in DLBCL is the IPI score [52]. Although it has been more recently suggested that it has lost predictive power following the introduction of rituximab, the IPI remains a valuable tool for risk stratification of DLBCL [33]. The improvement made in DLBCL classification into different molecular subtypes and the availability of treatment options point out the need for biomarkers that would better assess patient prognosis and therapy response.

Except for BCL6, the potential use of Oct2, IRF8, OCAB and PU.1 as prognosis biomarkers, has never been explored before in DLBCL. Among Oct2, BCL6 and IRF8, found highly expressed in our patients´ cohort, IRF8 showed an outstanding performance as a prognosis biomarker in the low risk group of patients that showed no bone marrow disease at diagnosis.

Regarding OCAB and PU.1 we found a reduced number of positive cases defined as those exhibiting more than 10% of stained tumor cell nuclei. Only 18 samples for OCAB and 11 for PU.1 showed more than 70% of positive tumor cells. Furthermore, 42.5% and 63% of the examined biopsies did not express OCAB or PU.1 respectively. We considered the low expression level or the negativity of the biopsies for these two TFs, as a loss of expression, since it is largely documented the high expression levels of both OCAB and PU.1 in normal B lymphocytes as they transit the GC phase of differentiation and maturation [5, 27].

The Kaplan-Meier study showed that PU.1 expression loss correlated with both DFS and OS applying a 70% cut-off point. In this regard, among relapsed patients during the follow-up period, 77% of them showed low PU.1 expression levels. When patients who died were considered, 74.2% showed low PU.1 expression levels at diagnosis. The PU.1 results strongly suggest that this TF might behave as a tumor suppressor in DLBCL patients.

In this regard, Rosenbauer et al demonstrated, using a mouse model, that decreased PU.1 expression levels resulted in various hematological malignancies development, including AML and B-CLL-like disease [58].

It has been suggested that PU.1 might be playing a role as a tumor suppressor in multiple myeloma [MM] and in classical Hodgkin Lymphoma [cHL] [59, 60].

Most human myeloma cell lines have lost PU.1 expression and patients primary myeloma cells have decreased PU.1 levels meanwhile normal plasma cells have relatively high levels. Tatetsu et al showed that PU.1 down-regulation in MM cell lines is caused by promoter hypermethylation [59].

It has been demonstrated that PU.1 induced expression in cHL cell lines triggered complete growth arrest and apoptosis. Furthermore a lentiviral system deliverying PU.1 induced apoptosis of primary cHL cells derived from patients. In addition, treatment of cHL cell lines with 5´-aza-2´-deoxycytidine and/or HDAC induced PU.1 expression, growth arrest and apoptosis in the cHL cell lines tested. These published data strongly supports the hypothesis of PU.1 role as a tumor suppressor in cHL where epigenetic modifications would be responsible for the gene silencing [59, 60].

OCAB co-factor behaved as PU.1 in terms of low and loss of expression. Although OCAB expression did not correlate neither with DFS or OS, we observed that 66.7% of the relapsed group of patients had low OCAB expression and 64.5% of patients that died also had low OCAB levels. In this regard, OCAB protein levels have been correlated with poor prognosis in hematopoietic malignancies before. Advani et al evaluated Oct2 and OCAB expression by IHC in patients with newly diagnosed AML [61]. Tomas et al studied Oct2 and OCAB protein expression in bone marrow tissue microarrays from patients with myeloma using fluorescent IHC, and correlated to patient survival. High Oct2 protein expression correlated with reduced survival whereas high OCAB protein expression correlated with increased survival [62]. Once again, these results are in accordance with the observations done in the DLBCL patients evaluated in this study.

The data we report sustain that the assessment of these five TFs expression levels reveal novel information on DLBCL patient prognosis and therapy response.

To date, clinical risk factors remain as the parameters taken into account for treatment decisions and prognosis. Biological prognostic factors and targeted therapies are, however, under active research and it is likely that patients will in the near future be treated according to risk-adapted and biomarker-driven therapies.

Acknowledgment

This work was supported by funds from the Agencia Nacional de Promoción Científica y Tecnológica [ANPCyT] through the Fondos para la Investigación Científica y Tecnológica [FONCyT]. PICT 00095/2012. Awarded to SMR.

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