The prognostic role of Bcl-2, Ki67, c-MYC and p53 in diffuse large B-cell lymphoma

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Abstract
Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of lymphoid malignancies, which counts for more than a third of non-Hodgkin’s lymphoma cases. The aim of the current study is to evaluate the prognostic role of several immunohistochemical (IHC) markers involved in the pathological process of DLBCL. This is a retrospective analysis of the 97 de novo DLBCL patients admitted between January 2007 and December 2016 in the Department of Hematology, “Filantropia” Municipal Hospital, Craiova, Romania. The expression of Bcl-2, Ki67, c-MYC and p53 was assessed by immunohistochemistry. A significant level of association was observed between high prognostic index values and Bcl-2, Ki67, c-MYC and p53 positive cases. Moreover, overall survival and disease-free survival were higher in patients with negative expression for these markers. Bcl-2, Ki67, c-MYC and p53 could make important diagnostic and therapeutic targets; therefore, their routine assessment should be mandatory.

Keywords: diffuse large B-cell lymphoma, immunohistochemistry, Bcl-2, Ki67, c-MYC, p53.

Introduction
Diffuse large B-cell lymphoma (DLBCL) is one of the most frequent lymphoid neoplasia, accounting for 30–40% of all cases of non-Hodgkin’s lymphomas, and displaying an aggressive course, a variable and unpredictable evolution that resides in its clinical and morphological heterogeneity [1–4].

The subtype of lymphoma is determined by the B-cell maturation stage and by the genetic abnormalities that occur during cell differentiation and maturation [5]. Although gene expression profiling has the highest accuracy identifying the “cell of origin” for certain lymphoma subtypes in DLBCL, it still has numerous limitations (various cellular regulating mechanisms, financial constraints, etc.). Therefore, a multitude of surface markers distinguishing germinal center B-cell like (GCB) DLBCL and activated B-cell like (ABC) DLBCL, that are consistent with gene expression profiling, have been tested. This is possible either by using the CD10, multiple myeloma oncogene-1 (MUM-1) and Bcl-6 biomarkers (the Hans algorithm) in 80% of cases, or using GCTE1, CD10, Bcl-6, MUM-1 and FOXP1 biomarkers (the Tally algorithm) in 90% of cases [6–8].

Furthermore, the detection of certain cell markers expression has become a fundamental component both in establishing an accurate prognosis, and in the development of an optimal therapeutic algorithm.

Materials and Methods

Case selection
A retrospective study was conducted on all de novo diffuse large B-cell lymphomas, diagnosed and treated in the Department of Hematology, “Filantropia” Municipal Hospital, Craiova, Romania, between January 2007 and December 2016, which revealed 128 newly discovered cases of DLBCL.

In case of IPI scoring system, a progressive scale from 0 to 5 was calculated by allocating one point for each individual parameter: clinical stage III/IV, age ≥60, the Eastern Cooperative Oncology Group (ECOG) score ≥2, extranodal lesions >1, and increased lactate dehydrogenase serum levels. Cases were included in two risk categories: low-risk group for patients with IPI 0–2, and high-risk group for patients with IPI>2.

This study aims to assess the prognostic value of four immunohistochemical (IHC) markers, other than those used in Hans protocol, by investigating the correlation between their expression and International Prognostic Index (IPI) score, as well as by comparing two important survival endpoints (disease-free survival and overall survival).
The presence of different cellular staining patterns (such as positive membrane, cytoplasmic or nuclear staining) was assessed in every slide. Each case was evaluated for the presence of different cellular staining patterns such as positive membrane, cytoplasmic or nuclear staining. The histological and IHC studies were performed on 97 formalin-fixed, paraffin-embedded tissue samples of the DLBCL patients admitted and treated in the Department of Hematology, “Filantropia” Municipal Hospital, Craiova.

Ethical approval for this study was acquired from the University of Medicine and Pharmacy of Craiova Ethical Research Committee.

### Histology and immunohistochemistry study

For the current study, we analyzed 97 formalin-fixed, paraffin-embedded tissue samples of the DLBCL patients admitted and treated in the Department of Hematology, “Filantropia” Municipal Hospital, Craiova.

The specimens were surgically or endoscopically retrieved for diagnostic confirmation and were preserved in the Department of Pathology, Emergency County Hospital, Craiova.

The paraffin-embedded tissue blocks were cut at 4 μm sections on a HM350 Microm rotary microtome (Thermo Fisher Scientific, Walldorf, Germany) with laminar water bath section transfer system (STS™, Thermo Fisher Scientific). For histological analysis, Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) trichrome stainings were subsequently performed.

For the immunohistochemical assessment, the histological sections were collected on poly-L-lysine covered slides that were dried in a thermostat, at 37°C for 24 hours to enhance the cell attachment to the plates. The sections subsequently underwent dewaxing, rehydration, antigen retrieval by boiling the histological slides in pH 6 sodium citrate buffer solution for 21 minutes (3 minutes cycles) in microwave oven. The slides were then rinsed in tap water and washed in distilled-deionized water for 15 minutes.

The endogenous peroxidase was blocked by immersing the slides in 3% hydrogen peroxide for 30 minutes, at room temperature, followed by rinsing in distilled water for 10 minutes, washing in 1% phosphate-buffered saline (PBS) for 5 minutes and blocking of non-specific sites with non-fat dry milk for 30 minutes. Next, the slides were incubated with primary antibodies for 18 hours, at 4°C. The following day, the sections were incubated for 30 minutes, at room temperature, with the secondary biotinylated antibody and washed in 1% PBS (three cycles of 5 minutes each). Streptavidine–HRP (Horseradish peroxidase) was added for 30 minutes, at room temperature, followed by washing in 1% PBS. The signal was detected by using 3,3'-Diaminobenzidine (DAB) (Dako, Agilent Pathology Solutions, Santa Clara, California, USA), and the reaction was blocked in 1% PBS. The next steps consisted of Mayer’s Hematoxylin counterstaining, alcohol dehydration and clearing with xylene. The coverslips were then mounted on DPX (Fluka, Honeywell Fluka™, Morris Plains, New Jersey, USA).

A brief description of the markers used in this study can be checked in Table 1.

### Statistical analysis

Data were investigated by descriptive statistics and logistic regression analysis. Five-year overall survival (OS) was defined as the interval in months from the initiation of therapy until death from any cause. All surviving patients or those who were drop out of the study at the end of the five-year follow-up were censored. Disease-free survival (DFS), as acknowledged by NCI Dictionary of Cancer Terms (https://www.cancer.gov/publications/dictionaries/cancer-terms), was defined as the time elapsed between primary treatment completion and any disease recurrence. Kaplan–Meier method was used to estimate the overall survival and disease-free survival probability, and survival curves were compared using both the Log Rank (Mantel–Cox) and Gehan–Wilcoxon regression methods. P-value less than 0.05 threshold was considered statistically significant. The statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, USA).

### Results

Subgroup correlations according to IHC pattern

The histological and IHC studies were performed on 97 tissue samples (85 cases of lymph node involvement, three cases of gastric lymphoma, two right colon tumors, one testicular tumor, three cutaneous “leg type” lymphoma, one maxillary tumor and two thoracic lymphomas). The specimens were taken from patients with a mean age of 56 years, undergoing diagnostic or radical oncological surgery in the Departments of Urology, Thoracic Surgery, General Surgery, and Maxillofacial Surgery, and treated in the Department of Hematology, “Filantropia” Municipal Hospital, Craiova.

Histopathological examination using HE and GS

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Code</th>
<th>Clone</th>
<th>Antigen retrieval</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Bcl-2</td>
<td>M0887</td>
<td>124</td>
<td>EDTA</td>
<td>Germinal center B-cells</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-Ki67</td>
<td>M7240</td>
<td>MIB-1</td>
<td>EDTA</td>
<td>Proliferation factor</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-c-MYC</td>
<td>ab32072</td>
<td>Y69</td>
<td>Sodium citrate buffer pH 6</td>
<td>Prognostic factor</td>
<td>1:250</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-p53</td>
<td>M7001</td>
<td>DO-7</td>
<td>Sodium citrate buffer pH 6</td>
<td>TP53 mutations</td>
<td>1:50</td>
<td>Dako</td>
</tr>
</tbody>
</table>

IHC: Immunohistochemical; DLBCL: Diffuse large B-cell lymphoma; EDTA: Ethylenediaminetetraacetic acid.
stainings revealed intense lymphoblastic cell proliferation, with round or oval shaped, hypochromic nuclei containing smooth inhomogeneous chromatin and 2–3 recognizable nucleoli (Figures 1 and 2). Sometimes, the nuclei displayed vesicular or pleomorphic architecture. The cytoplasm was light acidophilic and poorly represented in centroblastic lymphomas, and basophilic in immunoblastic lymphomas. The cells were usually round or oval shaped, but polyhedral cells have also been identified. Furthermore, numerous mitoses have been observed. Cellular proliferation led to the disruption and disorganization of the tissues. In the lymph nodes, the differences between the cortex and medulla were dimmed by the effacement of both the lymphoid follicles and medullary sinuses, as the entire nodal structure was replaced by a mostly monomorphic cellular population. At the gastric level, the mucosa was intensely infiltrated by lymphomatous cells, with the destruction of the glandular structures, muscularis mucosa, submucosa, and the invasion of the muscularis propria.

In terms of IHC reaction, an intense reaction to the anti-Bcl-2 antibody was observed in about 50% of cases (Figure 3), indicating an overexpression of this marker in tumoral cells. Bcl-2 is an antiapoptotic protein which inhibits the evolution of programmed cellular death, and its overexpression indicates an increase of genic amplification and translocation processes, that are common in DLBCL. In order to assess the proliferative activity of the tumoral cells, we used the anti-Ki67 antibody, which is the marker for a nuclear non-histone protein synthesized at the beginning of the cellular proliferation cycle. In our study, more than half of the cases presented with an intense IHC reaction to anti-Ki67 antibody (Figure 4).

One of the most frequently affected genes in the tumor proliferation process is TP53 gene, which is considered as the “guardian of the genome”, because it identifies and repairs the DNA lesions. The immunohistochemical expression of this gene is the p53 protein, which is detected by the anti-p53 antibody. The mutations in the TP53 gene are characterized by multiple molecular alterations associated with tumor development process and, more importantly, by the occurrence of wild p53 proteins, which can be identified by immunohistochemistry. In our study, the IHC reaction was positive in about 25% of cases (Figure 5).
c-MYC nuclear phosphoprotein is a transcription factor encoded by c-myc gene, which is involved in cellular growth, differentiation and apoptosis, making it one of the key molecules in tumorigenesis. Almost 32% of cases had a positive expression for c-MYC.

Table 2 indicates the immunophenotypic features of the 97 patients with de novo DLBCL, according to their cell-of-origin distribution. Forty-four (45.36%) cases were GCB DLBCL and 53 (54.64%) cases were non-GCB lymphomas. Bcl-2 displayed similar distribution in both subgroups (positivity in 41.67% of cases for GCB and in 58.33% of non-GCB patients, p=0.05). Likewise, no correlations could be observed for p53 expression. However, a strong correlation between c-MYC+ expression and non-Bcl subtype was observed (p<0.005, Odds ratio – OR=4.374), with similar results found in Ki67 expression (p=0.001, OR=7.382).

When the IPI distribution across the group was investigated, significant correlations were observed between high prognostic index values (>2) and positive expression for all four markers (Table 3).

Table 2 – Immunophenotypic features of DLBCL patients according to the cell-of-origin subtype

<table>
<thead>
<tr>
<th></th>
<th>Bcl-2</th>
<th>Ki67</th>
<th>c-MYC</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>GCB</td>
<td>49 (50.5)</td>
<td>48 (49.5)</td>
<td>40 (41.23)</td>
<td>57 (58.77)</td>
</tr>
<tr>
<td>Non-GCB</td>
<td>25</td>
<td>28</td>
<td>11</td>
<td>42</td>
</tr>
<tr>
<td>P-value (Fisher)</td>
<td>0.5426</td>
<td>&lt;0.001</td>
<td>0.023</td>
<td>0.0616</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.344 (0.5847–2.888)</td>
<td>7.382 (3.011–17.72)</td>
<td>4.374 (1.643–10.86)</td>
<td>2.718 (1.058–6.841)</td>
</tr>
</tbody>
</table>

Table 3 – Association between the IPI and the immunohistochemical profile of DLBCL patients

<table>
<thead>
<tr>
<th></th>
<th>Bcl-2</th>
<th>Ki67</th>
<th>c-MYC</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IPI 0–2</td>
<td>30</td>
<td>18</td>
<td>29</td>
<td>42</td>
</tr>
<tr>
<td>IPI 3–5</td>
<td>19</td>
<td>30</td>
<td>11</td>
<td>38</td>
</tr>
<tr>
<td>P-value (Fisher)</td>
<td>0.0257</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>2.632 (1.131–5.995)</td>
<td>5.273 (2.238–12.98)</td>
<td>7.292 (2.56–20.16)</td>
<td>5.088 (2.037–11.72)</td>
</tr>
</tbody>
</table>

IPI: International Prognostic Index; DLBCL: Diffuse large B-cell lymphoma; OR: Odds ratio; CI: Confidence interval.

Discussion

DLBCL is one of the most frequently encountered types of aggressive lymphoma, so sustained efforts have been made to find the relevant features involved in its clinical behavior and therapeutic response [9]. A long list of clinical, demographic, topographical, and histological factors have been investigated, but most of the results were insubstantial [10, 11]. DLBCL can affect patients of all ages, with a wide range of clinical manifestations. However, mean age at presentation is 64 years, and the disease has a slightly higher incidence in male patients. DLBCL can involve any organ, including the central nervous system, but 50% of cases are diagnosed in the advanced stages (stages III and IV Ann Arbor) [12, 13].
The prognostic role of Bcl-2, Ki67, c-MYC and p53 in diffuse large B-cell lymphoma

Figure 6 – Overall survival according to Bcl-2 expression in DLBCL population (p=0.06, Mantel–Cox test; p=0.0501, Gehan–Wilcoxon test).

Figure 7 – Disease-free survival according to Bcl-2 expression in DLBCL population (p<0.001, Mantel–Cox test; p<0.001, Gehan–Wilcoxon test).

Figure 8 – Overall survival according to p53 expression in DLBCL population (p=0.0034, Mantel–Cox test; p=0.0069, Gehan–Wilcoxon test).

Figure 9 – Disease-free survival according to p53 expression in DLBCL population (p=0.0175, Mantel–Cox test; p=0.0269, Gehan–Wilcoxon test).

Figure 10 – Overall survival according to c-MYC expression in DLBCL population (p<0.001, Mantel–Cox test; p<0.001, Gehan–Wilcoxon test).

Figure 11 – Disease-free survival according to c-MYC expression in DLBCL population (p<0.001, Mantel–Cox test; p<0.001, Gehan–Wilcoxon test).

The role of immunophenotype variability for the therapeutic outcome has long been the cornerstone for DLBCL management strategy. It is important to know from the beginning how to approach a patient whose therapy is probable to fail, in order to achieve a complete response with standard chemotherapy because in this category of patients the outcome is unfavorable. Thus, it is necessary to use an effective, well-adapted first line or salvage therapy when taking into consideration the prognosis factors [14].

With the help of molecular biology, progress has been made in the understanding of non-Hodgkin’s lymphomas pathogenesis, by finding new oncogenic pathways with various treatment targets. In the last few years, new treatment approaches like targeted agents are increasingly studied as a single agent or in combination with other chemotherapeutic drugs. Currently, patients are still treated in a uniform manner, and the discovery of predictive biomarkers will allow a personalized form of treatment. Only 40–50% of the patients obtain a sustained
response with classical R-CHOP, therefore it is important to establish from the moment of diagnosis if it is necessary to apply a more aggressive or even an experimental treatment. The process through which this can be achieved is by independently assessing the morphological, IHC and genetic profile of every patient who will be subsequently selected for a more individualized treatment.

For the current analysis, the morphological classification of cases was performed in compliance with the 2008 WHO classification. We considered the following morphological subtypes:

- Immunoblastic – when immunoblasts accounted for 90% or more of the tumor cells;
- Centroblastic – when centroblasts represented 90% or more of the neoplastic cells; and
- Anaplastic subtype – roughly characterized by round cells with bizarre, pleomorphic nuclei.

The Hans protocol was used for DLBCL subdivision into GCB and non-GCB type. Positive expression for CD10 or co-expression of CD10 and Bcl-6 allowed the distribution of cases to the GCB group, whereas negative expression of CD10 with positive Bcl-6 dictated for the evaluation of MUM-1. The negative expression of MUM-1 confirmed the inclusion in the GCB group.

The role of this study was to determine how several molecular markers (Bcl-2, Ki67, c-MYC and p53) not included in Hans algorithm may influence the survival endpoints and thus correlate with the prognosis. In order to have unequivocal and reproducible results, single cutoff values were attributed to each of these parameters. We assessed each case as positive if 30% or more of the tumor cells were stained with an antibody, in accordance with the criteria recommended by Hans et al. [8]. We immunohistochemically analyzed the expression of Ki67 in tissue sections by considering a cut-off value of 70%. The cutoffs used for Bcl-2, c-MYC and p53 were 70%, 40% and 30%, respectively.

As a routinely determined marker necessary in Hans algorithm, Bcl-6, along with the other specific markers needed for cell-of-origin classification, such as CD10 and MUM-1, can serve as selective diagnostic and therapeutic target for GCB DLBCL. As there are several studies that already investigated this specific aspect [15, 16], more efforts should be made towards exploring new, insufficiently investigated markers.

One notable example is Bcl-2, an important regulating protein that is heterogeneously expressed in both GCB and non-Bcl DLBCL, by distinct mechanisms. In GCB DLBCL, (t(14,18) translocation is involved, unlike non-GCB DLBCL where gene amplification and transcriptional upregulation are responsible for its expression. GCB DLBCL expressing Bcl-2 present a low response to the classical R-CHOP treatment compared to the non-GCB DLBCL, therefore Bcl-2 inhibitors are also under investigations [17, 18].

Up to 30% of DLBCL cases express c-MYC protein, which is involved in growth and cell proliferation. Expression of c-MYC seems to be uncorrelated with poor prognosis, instead co-expression of c-MYC and Bcl-2 appear to signal a low overall survival and disease-free survival [19]. Moreover, c-MYC–Bcl-2 co-expression seems to have a higher incidence in non-Bcl cases, and appear to bear a higher importance to prognosis than the cell-of-origin classification in itself [20]. Based on these characteristics, patients with DLBCL should be routinely investigated for the expression of these two molecules with prognosis importance, so that different therapy could be considered in this category of patients.

P53, on the other hand, plays a controversial role as a prognostic marker, as the results of several studies investigating this aspect are discordant. There might be several explanations for this phenomenon, such as low cutoff values for IHC expression, small sample size, heterogeneous population or different treatment protocols [20, 21]. No such inconsistencies were noted in our group, as the sample size was adequate for proper statistical analysis and only a single, routinely used treatment regimen was considered (R-CHOP). Moreover, a high cutoff value for p53 (>30%) was used for more consistent results. Nevertheless, positive expression of p53 was correlated with worse results in terms of overall survival and disease-free survival, which is consistent with the results of other studies.

Ki67 is a nuclear marker that signalizes the lymphoproliferative activity of the non-Hodgkin’s lymphoma. Therefore, its expression is routinely marked as an index of proliferation, with potential prognostic value for various DLBCL subtypes. Despite numerous attempts to correlate its expression to other biological markers or to certain clinical and therapeutic outcomes, no definitive conclusions could be delivered [22–24]. Similar to the level of the IHC expression of the other three markers, our results for Ki67 are unequivocal, providing a good argument for their routine use as useful additions to the diagnostic protocol and treatment algorithm of DLBCL.

However, our study has several limitations, due to the reduced number of patients and its retrospective design, thus the need for the initiation of larger, prospective randomized controlled trials in order to have a definitive and clear view of the role these markers play in the clinical outcome of DLBCL patients.

Conclusions
The results of our study suggest that the positive expression of Bcl-2, Ki67, c-MYC and p53 positively correlates with low prognostic index and poor survival rate, thus making them valuable diagnostic and therapeutic targets, hence the need for their routine evaluation as part of a new IHC algorithm for DLBCL.

Conflict of interests
The authors have no conflict of interests to disclose.

Author contribution
Ana-Maria Pătraşcu and Ionela Rotaru had equal contribution to this study thus share main authorship.

References
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