Doctoral (Ph.D.) thesis

Clinical follow up of cytogenetically subclassified diffuse large B-cell lymphoma (DLBCL)

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1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive neoplasm of B-lymphocytes. DLBCL is the most common subtype of high-grade non-Hodgkin lymphoma (NHL) accounting for 30-40% of B-cell NHLs and for 80% of aggressive lymphomas. DLBCL has considerable biologic, molecular, and clinical heterogeneity resulting in different responses to therapy. This heterogeneity is also reflected in the latest classification of the World Health Organization (WHO) published in 2023. To establish the diagnosis, histological, immunohistochemical (IHC) and fluorescent in situ hybridization (FISH) examination of an excision sample or core biopsy is required.

Several prognostic scoring systems, that include clinical parameters, have been developed to assist risk stratification and treatment decisions, namely the International Prognostic Index (IPI), the revised IPI (R-IPI) and the National Comprehensive Cancer Network IPI (NCCN-IPI). However, the accurate identification of very high-risk patients is not accomplished by these scores. In recent years much more detailed classification of DLBCL subtypes has become possible, based on the pattern of genetic abnormalities and the results of gene expression studies. The profound molecular heterogeneity of DLBCL is the underlying reason why up to 45%–50% of patients develop relapsed/refractory disease, which remains the major cause of mortality.

The original DLBCL molecular classification using DNA microarray-based technology was initially described by Alizadeh et al. in their landmark study. This technology distinguished two major cell of origin (COO) subtypes of DLBCL: germinal center B-cell like (GCB) and activated B-cell like (ABC). The ABC subgroup has much poorer prognosis compared to the GCB group when treated with the standard immunochemotherapy. The possibility to perform GEP on fresh frozen samples is very limited. The results of defining the cell of origin by immunohistochemical methods have been shown good correlation with the GEP results. The immunohistochemical algorithm developed by Hans has become the most common method to determine COO using three markers: CD10, BCL6 and MUM1.

The 50-60% of the DLBCL patients can be cured by the standard R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone) immunochemotherapy. Patients who receive first-line R-CHOP immunochemotherapy and who have not an event at 24 months from diagnosis (event-free survival 24, EFS24) have excellent outcomes with an overall survival (OS) that is similar to the age- and sex-matched

general populations. The results of our study group (Ritter et al.) confirmed the predictive role of EFS24 based on in vivo clinical and radiomic parameters in DLBCL patients.

Beyond the immunochemotherapy in many NHL cases, the radioimmunotherapy (RIT) can be a promising therapeutic option. The most commonly used the ^{90}yttrium-labeled ibritumomab tiuxetan (^{90}YIT) consists of an anti-CD20 murine monoclonal antibody conjugated with a radioactive isotope purely emitting beta particle. The current guidelines of the European Society for Medical Oncology (ESMO) do not mention RIT as a therapeutic option for DLBCL. Our working group (Szakács et al.) conducted a single-arm, retrospective cohort study on NHL patients who received RIT in regional hospitals between 2004 and 2008 based on the data from the United Arab Emirates lymphoma registry. The indication of RIT was far broader than that approved by the FDA because of the limited availibility of the autologous stem cell transplantation. In second or third line setting (mostly in DLBCL patients) the median EFS was 33 months. Our results suggest that patients with B-cell NHL treated with ^{90}YIT experience satisfactory OS and EFS with acceptable safety profile.

2. Aims of the study

We set as a goal the immunohistochemistry and interphase FISH examination of the tissue samples of DLBCL patients diagnosed at the Department of Pathology, University of Pécs treated at various haematological centers in the country. We planned to isolate DNA and RNA from formalin-fixed, paraffin-embedded (FFPE) tissue blocks for next generation sequencing (NGS)-based genomic and NanoString-based expression profile studies to be performed by other working groups. Parallel with the pathological examinations we aimed to investigate the clinical and survival data of the patients. The main goal of our study was to evaluate the role of the IPI and molecular biomarkers, and to create a prognostic model that can be easily applied clinically to estimate the event-free and overall survival. Our aim was to determine the proportion of DLBCL patients stratified to the GCB and non-GCB subtypes using the immunohistochemical data based on the Hans algorithm, determine their prognosis, and compare our findings with international research results.

3. Patients and Methods

This multicenter, retrospective study was approved by the Committee of Science and Research Ethics (ETT-TUKEB) under reference number 50268-8/2017.

A total of 342 adult patients diagnosed with DLBCL were assessed. Tissue samples were sent by 7 Hungarian Hematology centers to the central hematopathology lab in the Department of Pathology, University of Pécs between Jan 2010 and Mar 2017. Out of the 342, 95 cases were excluded due to incomplete clinicopathological data, so a total of 247 cases were further assessed in the study.

We investigated the FFPE blocks of patients with high grade B-cell NHL previously not treated. All patient data were analyzed by immunohistochemistry for CD10, BCL2, BCL6, MUM1 and MYC expression. The proliferation index was defined using MIB1 antibody-based criteria. Interphase FISH testing was performed for *MYC::IgH, IgH::BCL2* translocation, *BCL6* break and for *MYC, BCL2* and *BCL6* amplification.

DNA was isolated from the FFPE tissue blocks for next generation sequencing (NGS)-based genomic studies performed by another working group. Expression profile tests were carried out in collaboration, with NanoString technology, in accordance with the Pan CancerProgression / SignalPathway platforms or with sense / antisense NGS procedures from the isolated RNA. Gene expression profiling was accomplished in 173 samples.

3.1 Immunohistochemistry

IHC was carried out according to standard protocols using CD10, clone 56C6; CD20, clone L26 (Visionbiosystems Novocastra, UK); MUM1/IRF4, clone MUM1p; BCL2, clone 124; BCL6, clone PG-B6p (Dako, Denmark); MYC, clone Y69 (Abcam, UK); Ki-67, clone B56 (Hisztopatológia Kft., Hungary) specific primary antibodies as well as Envision+ System-HRP (DakoCytomation, Denmark) and Bond Polymer Refine Detection (Leica Biosystems, UK) developing reagents. According to the Hans algorithm, at least 30% reactivity–either membranous or nuclear–is required for a tumor to be considered positive for a particular marker. The tumors were classified as GCB-like when exhibiting CD10+ (BCL6+/-, MUM1+/-) or CD10-, BCL6+, MUM1- phenotype. DEL was defined as combined BCL2 (≥50%) and MYC (≥40%) positivity.

3.2. Interphase FISH

Interphase FISH was performed using 5 μm paraffin tissue sections for *IGH::MYC, IGH::BCL2, BCL6* rearrangement and for *MYC, BCL2, BCL6* gene copy number (GCN) gain. For these, Vysis IGH/MYC/CEP8 TC-DF, Vysis LSI IGH/BCL2 DC-DF, and Vysis LSI BCL6 (ABR) DC Break Apart probes (Abbott Molecular Inc., USA) were used. FISH reactions were analyzed in Zeiss Axioplan-MOT II fluorescent microscope and evaluated by means of 'grid sampling' and 'color rationing' methods. We have used double fusion FISH probes to detect *IGH::BCL2* and *IGH::MYC* fusions, since non-*IG BCL2* fusions are rare in DLBCL, and the prognostic significance of non-*IG MYC* fusions is controversial. Cases with non-*IG BCL2* or *MYC* fusions showed signal patterns indicating *BCL2* or *MYC* gain in our series. A tumor was defined positive for rearrangement using IGH/MYC, IGH/BCL2, and BCL6 probes if the fusion or the dissociated FISH signs occurred in at least 50% of the nuclei.

3.3. Clinical assessment

Detailed clinical and laboratory data including treatment regimen and clinical outcomes (OS, EFS, EFS24) were collected from patients' records, then, all data were reviewed by a senior hematologist. The clinical stage was evaluated by the modified Ann Arbor and Lugano classifications. Complete response, partial response, progression, refractory disease, and relapse were defined according to the International Working Group response criteria for lymphoma. EFS was defined as the time from the end of first line treatment until the earliest occurrence of disease progression or death of any cause. EFS24 was defined as being alive and free of any disease related event 24 months from the end of therapy.

3.4. Statistical analysis

In univariate statistics, Chi^2 test was used to analyze the association across clinical variables. Kaplan-Meier curves with a median estimate (with 95% confidence interval, CI) and the log-rank test were used for univariate survival analysis. Multivariate Cox regression analysis was applied to identify independent prognostic factors for the outcomes (OS and EFS). The models were adjusted for gender, IPI subgroups, IHC (CD10, BCL6, MUM1, high MIB-1 >90%, MYC, and BCL2) and for FISH findings (*BCL6* translocation and *BCL2* GCN gain). In general, $P < 0.05$ value was considered statistically significant. Statistical analysis

was performed using R statistical software version 4.2.0 and the survminer package v0.4.9 statistical software.

4. Results

4.1. Characteristics of patients

The median age at the time of diagnosis was 65 years (range: 19–91 years), 65.2% of patients were >60 years, 46.6% were male, 42.9% had an elevated serum lactate dehydrogenase (LDH) and 74.1% had an advanced (stage III/IV) disease. The majority (94.7%) were treated with R-CHOP or similar regimens.

4.2. Immunohistochemical results

A total of 234 patients had available IHC data, respectively. A positive IHC staining for MYC, BCL2, BCL6, and MUM1 protein was seen in 52.1%, 66.2%, 72.6%, and 77.8%, respectively. DEL (MYC and BCL2 co-expression) accounted for 33.3% and did not occur more frequently in the non-GCB group (p=0.112). High proliferation index (MIB-1 antibody >90%) was detected in 26.2%. Based on the Hans algorithm, non-GCB and GCB types accounted for 63.7% (149/234 cases) and 36.3% (85/234 cases), which classification corresponded in 82.6% to the genome expression profile stratification.

4.3. Interphase FISH results

A total of 220 patients had available FISH data, respectively. *MYC* translocation was detected in 16 cases (7.3%), and all of them were positive for MYC protein expression. There were only 4 cases (1.8%) of MYC GCN gain.

BCL2 translocation was detected in 7.3%, all were in the GCB group (p<0.001). *BCL2* GCN gain was detected in 14.1% of cases. *BCL6* gene rearrangement was confirmed in 21.4%, and it was significantly associated with the non-GCB subtype (p=0.006). There were only 2 cases with *BCL6* GCN gain. There were 4 cases (1.8%) of dual *MYC* and *BCL2* translocations (2 cases of DEL, another two had only BCL2 protein overexpression), and all were in the GCB group. Triple hit lymphoma did not occur.

4.4. Survival

At a median follow-up of 52 months (range: 0–131 months), 140 patients (56.7%) had disease progression or relapse. The overall response rate was 78.4% and the CR rate was 47.0%. The Kaplan-Meier estimate for EFS24 was 56.2% (CI: 50.4–62.8%). Five-year OS was 45.4% (CI:39.5-52.1%). BCL6 expression and low IPI score were significant positive predictors of OS and EFS in univariate analysis, whereas MUM1 predicted negatively only EFS. *BCL6*

rearrangement, *BCL2* GCN gain, *IGH::MYC* translocation, and *IGH::BCL2* translocation did not have any prognostic impact on survival. Subgroup analysis by COO did not change the findings, nor did we find any difference in OS and EFS by COO subtype. Our results showed no difference in the 5-year survival in low-stage (I-II) and high-stage disease according to the COO. DEL phenotype did not predict OS or EFS. We did not find any impact of double protein expression using MYC and BCL2 on the OS in low and high-stage diseases.

Table 1 summarizes the findings of the multivariate analysis of 220 patients. IPI score was a significant independent negative, whereas MIB-1 and BCL6 protein expressions were significant independent positive predictors of both OS and EFS.

Table 1. Multivariate Cox regression analysis for overall survival and event-free survival

	Overall survival			**Event-free survival**		
	HR	**CI**	**P-value**	**HR**	**CI**	**P-value**
Gender	0.723	0.501–1.106	0.094	0.817	0.551–1.212	0.315
IPI						
IPI 1	ref.			ref.		
IPI 2	4.732	1.952–11.474	<0.001*	3.698	1.502–9.106	0.004*
IPI 3–5	10.451	4.515–24.193	<0.001*	8.600	3.702–19.976	<0.001*
CD10 expression	1.581	0.975–2.566	0.063	1.626	0.975–2.713	0.063
BCL6 expression	0.649	0.425–0.990	0.045*	0.623	0.398–0.976	0.039*
MUM1 expression	1.194	0.694–2.053	0.523	1.454	0.789–2.679	0.230
MIB-1>90%	0.581	0.364–0.927	0.023*	0.597	0.367–0.971	0.038*
MYC expression	1.071	0.724–1.585	0.732	1.141	0.758–1.719	0.528
BCL2 expression	0.993	0.656–1.503	0.973	0.952	0.6183–1.466	0.824
***BCL6* translocation**	0.967	0.617–1.514	0.883	1.057	0.667–1.676	0.813
***BCL2* GCN gain**	0.984	0.589–1.643	0.950	1.105	0.649–1.882	0.713

For molecular markers, the reference group is always the ’negative’ group. Asterisks indicate statistical significance.

Abbreviations: IPI, International Prognostic Index; ref, reference; GCN, gain copy number

5. Discussion

In the current study, we used IHC and FISH techniques on samples from DLBCL patients treated with rituximab and we defined the COO subgroups based on the Hans algorithm. In our cohort of patients, the COO phenotype failed to predict prognosis, which is surprising knowing that some studies have demonstrated significantly better survival for the GCB group.

Factors that were independently associated with OS and EFS in the multivariate analysis were IPI, high MIB-1 (>90%), and BCL6 expression. BCL6 protein overexpression carries a positive prognostic effect on OS and EFS. The high proliferation index (>90%) detected by MIB-1 antibody was proved to be an independent predictor of good prognosis regarding OS and EFS. There are still significant differences in OS and EFS across the IPI groups even in the rituximab era. Our study has several strengths and limitations. The major strength of our work includes the size and coverage of the study population (247 DLBCL cases from 7 Hungarian centers), allowing a detailed and representative survival analysis. The major limitation of our work is the study's retrospective design, which, as reflected by the number of excluded patients, resulted in lack of a full dataset in some analyses. These findings may improve prognostication in DLBCL and can contribute to designing further research in the area. However, considering the limitations of our study, these findings should be validated in prospective series.

6. Summary of the new results

1. Our study is the first Hungarian multicenter, representative study including a large number of DLBCL patients, which, processes the immunohistochemical and cytogenetic data that are the basis of prognosis and evaluates the detailed survival data of the patients.

2. Our study confirms the independent positive predictive value of BCL6 expression in DLBCL in post-rituximab era for both overall and event free survival. Our study provides strong basis for designing prospective research on the prognostic significance of B-cell differentiation genes encoding proteins in DLBCL.

3. To the best of our knowledge, this is the first study that shows positive predictive value of high MIB-1 expression on the outcomes of DLBCL treated with rituximab based immunochemotherapy.

4. Our studies confirm the predictive value of EFS24 based on in vivo clinical and radiomic parameters in DLBCL patients.

5. In a unique study, our working group confirmed the effectiveness of RIT in relapsed or refractory DLBCL in cases when autologous stem cell transplantation is not available. This therapeutic modality is currently not indicated for DLBCL in Hungary. Based on our results, RIT could be a potential therapeutic option for patients with refractory or relapsing DLBCL who do not accept or are not suitable for autologous stem cell transplantation.

Publications

Publications related to the thesis

A Balikó, Z Szakács, B Kajtár, Z Ritter, A Gyenesei, N Farkas, L Kereskai, I Vályi-Nagy, H Alizadeh, L Pajor (2023) Clinicopathological Analysis of Diffuse Large B-cell Lymphoma Using Molecular Biomarkers: A Retrospective Analysis from 7 Hungarian Centers. Front. Oncol. 13:1224733. doi: 10.3389/fonc.2023.1224733 (Q1, IF: 5.738)

Szakács Z, Lal A, Kristensen J, Farkas N, Ritter Z, Kiss S, Alizadeh H, **Balikó A.** ^{90}Y-ibritumomab Tiuxetan in B-cell Non-Hodgkin Lymphomas: Real-world Data From the United Arab Emirates. Adv Radiat Oncol. 2021 Dec 26;7(5):100882. doi: 10.1016/j.adro.2021.100882 (Q1, IF: 3.08)

Ritter Z, Papp L, Zámbó K, Tóth Z, Dezső D, Veres DS, Máthé D, Budán F, Karádi É, **Balikó A,** Pajor L, Szomor Á, Schmidt E, Alizadeh H. Two-Year Event-Free Survival Prediction in DLBCL Patients Based on *In Vivo* Radiomics and Clinical Parameters. Front Oncol. 2022 Jun 8;12:820136. doi: 10.3389/fonc.2022.820136. (Q1, IF: 5.738)

Pajor, L., Kun, J., Herczeg, R., Gálik, B., Ritter, Z., **Baliko, A**., Hegedűs, G., Barna, T., Czifra, J., Pálvölgyi, A., Östör, G., Babarczi, E., Ligeti, E., Kránitz, N., Bali, O., Bodor, A., Tiszlavicz, L., Pajor, G., Kajtár, B., Kereskai, L., Tornóczki, T., Alizadeh, H., Gyenesei, A., & Vályi-Nagy, I. (2023). A diffúz nagy B-sejtes limfóma fenotipikus, citogenetikai és expressziós profil heterogenitása – Magyarországi multicentrikus tanulmány, *Hematológia–Transzfuziológia*, *55*(4), 154-163.

Publication not related to the thesis

A. Mikó, R. Csalódi, S. Kosztolányi, Á. Nagy, Á. Szomor, O. Tóth, J. Pammer, Z. Kohl, E. Sziládi, **A. Balikó,** and H. Alizadeh, "Drug-induced thrombotic microangiopathy caused by ponatinib," EUROPEAN JOURNAL PHARMACEUTICAL AND MEDICAL RESEARCH, vol. 6, no. 5, pp. 589–595, 2019.

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