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ORIGINAL ARTICLE

The Role of Lymphocyte to Monocyte Ratio, Microvessel Density and HiGH CD44 Tumor Cell Expression in Non Hodgkin Lymphomas

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Abstract Prognostic significance of immune microenvironment has been emphasized using the most advanced analysis, with consecutive attempts to reveal prognostic impact of this findings. The aim of this study was to compare and define prognostic significance of clinical parameters, microvessel density (MVD) in tumour tissue and expression of CD44s as adhesive molecule on tumour cells in diffuse large B cell lymphoma-DLBCL, primary central nervous system DLBCL-CNS DLBCL and follicular lymphoma-FL. A total of 202 histopathological samples (115 DLBCL/65 FL/22 CNS DLBCL) were evaluated. Overall response (complete/partial remission) was achieved in 81.3 % DLBCL patients, 81.8 % primary CNS DLBCL and 92.3 % FL. Absolute lymphocyte count-ALC/Absolute monocyte count-AMC >2.6 in DLBCL and ALC/AMC≥4.7 in FL were associated with better event-free survival (EFS) and overall survival (OS) ($p<0.05$). In DLBCL, MVD>42 blood vessels/0.36 mm^2 correlated with primary resistant disease ($p<0.0001$), poorer EFS and OS ($p=0.014$). High CD44s expression in FL correlated with inferior EFS and OS ($p<0.01$). In DLBCL, multivariate Cox regression analysis showed that ALC/AMC was independent parameter that affected OS (HR 3.27, 95 % CI 1.51–7.09, $p=0.003$) along with the NCCN-IPI (HR 1.39, 95 % CI 1.08–1.79, $p=0.01$). Furthermore, in FL, ALC/AMC mostly influenced OS (HR 5.21, 95 % CI 1.17–23.21, $p=0.03$), followed with the FLIPI (HR 3.98, 95 % CI 1.06–14.95, $p=0.041$). In DLBCL and FL, ALC/AMC is simple and robust tool that is, with current prognostic scores, able to define long-term survival and identify patients with inferior outcome. The introduction of immunochemotherapy might altered the prognostic significance of microenvionmental biomarkers (MVD and CD44s).

Keywords Lymphoma · Lymphocyte-to-monocyte count · Adhesion · Angiogenesis

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Introduction

Among non-Hodgkin lymphomas (NHL), diffuse large B cell lymphoma (DLBCL) represents the most common type with approximately 20–30 % of all NHL [1]. Primary central nervous system (CNS) lymphoma represents less than 1 % of all NHL with majority of cases (90 %) that belong to DLBCL (CNS DLBCL), however low grade lymphomas and Burkitt lymphoma can also be present in CNS [2]. Furthermore, follicular lymphoma (FL) constitutes about 20 % of NHL, with significantly lower incidence in Eastern Europe [2]. According to the gene expression profiling (GEP), two DLBCL different prognostic subtypes, including germinal centre B cell (GCB) and activated B cell (ABC) have been identified [3]. However, the lack of availability of GEP in a

routine clinical practice and unsatisfactory correlation with immunohistochemical algorithms, has pointed out the need of recognition of additional markers [2]. Furthermore, GEP and immunohistochemistry based studies in NHL have emphasized the role of tumour microenvironment (tumour associated macrophages) and host immune homeostasis (tumour infiltrating lymphocytes) in lymphoma development which has resulted in recognition of the prognostic importance of absolute lymphocyte count (ALC) at diagnosis, absolute monocyte count (AMC) and possibly lymphocyte-to-monocyte ratio (ALC/AMC) [4–11]. According to the GEP analysis, majority of primary CNS DLBCL belong to the unfavourable non-GCB subtype, however the role of microenvironment in CNS is still unclear [12–14]. Since the introduction of rituximab, the outcome of DLBCL and FL has been significantly improved, conversely the outcome of primary CNS DLBCL still remains poor [15–17]. The value of previously developed prognostic scores has diminished in addition to the prognostic impact of immunohistochemical markers, thus the revaluation is needed [18–20].

In lymphoma tumour tissue, microvessel density (MVD) is the highest in aggressive subtypes of lymphoma, including Burkitt's lymphoma and peripheral T-cell lymphoma, compared with intermediate in DLBCL and lower in indolent FL [21]. Some studies have reported that higher MVD was associated with poor overall survival (OS), while others have suggested no correlation of MVD with the International Prognostic Index (IPI) and clinical outcome in patients treated with chemotherapy [21]. Nevertheless, in the era of immunochemotherapy, validation of prognostic impact of angiogenesis is still obscure [21]. Furthermore, in primary CNS DLBCL, accumulation of lymphoma cells around vessels is often found, but the prognostic implication of this histopathological pattern is not well established [17]. Tumour angiogenesis is mediated with a range of different molecules including antigen CD44 on tumour cells which is part of the family of glycoprotein adhesive molecules that also mediate tumour invasion and metastasis [22, 23]. Standard form of CD44 (CD44s) is ubiquitously expressed and number of its variants are detected on malignant cells [24, 25]. Higher CD44s expression has been noticed in non-GCB DLBCL compared to GCB DLBCL, but contradictory data have been also reported [24, 25]. Furthermore, majority of studies were performed on the DLBCL patients treated with chemotherapy, and only few of them investigated the impact of CD44s in the rituximab era suggesting potential altered prognostic significance of CD44s expression [25]. Also, higher CD44s was reported in FL with diffuse histopathological pattern [26].

The aim of this study was to analyse simple and widely available tools in NHL, such as ALC/AMC, MVD and CD44s expression on tumour cell, which even though altered by rituximab introduction, are capable to identify high risk patients with current prognostic scores.

Materials and Methods

The pre-therapy formalin fixed, paraffin wax embedded (FFPE) samples of 202 patients (95 males/107 females) diagnosed between 2004 and 2013 at Clinical Centre of Serbia were collected including 22 cases of primary CNS DLBCL (10.9 %), 49 cases of nodal DLBCL (24.3 %), 66 cases of extranodal DLBCL (33.7 %) and 65 cases of FL (33.2 %) [2]. In addition, the samples of immunodeficiency-associated tumours, primary effusion and primary mediastinal lymphoma were excluded. All patients underwent standard staging procedures in order to define the disease stage. All laboratory tests, including ALC and AMC were measured at diagnosis previous to chemotherapy. The detailed clinical characteristics of analysed patients are listed in the Table 1.

Therapy response was evaluated according to Cheson criteria [27]. Treatment included rituximab plus CHOP/CHOP like based combinations (cyclophosphamide, doxorubicin, vincristine, and prednisolone) applied in 180 patients with DLBCL and FL and high doze methotrexate (HD-MTX) with/without whole brain irradiation (WBRT) in 22 primary CNS DLBCL. All primary CNS DLBCL patients were firstly admitted to neurosurgery department, where initial diagnostic and therapeutic procedures were performed based on clinical and radiography data. Complete tumour resection, without tumour rest on control computed tomography scan, previous to HD-MTX was performed in 10 primary CNS DLBCL patients (45.5 %), while seven patients (31.8 %) had partial tumour resection and 5/22 (22.7 %) had tumour biopsy only [28]. The study was performed according to the principals of the Declaration of Helsinki and was approved by the Research Ethics Board of the Faculty of Medicine, University of Belgrade.

Tissue Microarray Construction

All tumour samples were reviewed by three pathologist, and diagnosis of lymphoma was set based on the 2008 WHO criteria [2]. Firstly, the samples were examined in order to select representative tumour areas and mark them in the paraffin blocks. In each case, three cylinders 1-mm in diameter were selected from different areas, along with controls. Then the procedure of constructing tissue microarrays (TMA) was done as previously reported [29]. Each sample was three times punched from the representative area for construction of the recipient block. Then the sections (4 μm thick) of these TMA blocks were transferred to an adhesive coated glass slide system. The samples that were not available for TMA were analysed as whole-tissue sections.

Table 1 Summarized clinical characteristics of 202 patients with diffuse large B cell lymphoma, primary central nervous system diffuse large B cell lymphoma and follicular lymphoma

	Nodal DLBCL N (%)	Extranodal DLBCL N (%)	Primary CNS DLBCL N (%)	FL N (%)	
Age, y, median (range)	58 (21–77)	60 (26–80)	53.5 (29–69)	54 (32–82)	
male/female	24/25 (49.0/50.0)	31/35 (47.0/53.0)	7/15 (31.8/68.2)	33/32 (50.8/49.2)	
Stage I-II	23 (46.9)	33 (50.0)		5 (7.7)	
Stage III-IV	26 (53.1)	33 (50.0)		60 (92.3)	
B symptoms: Yes	35 (71.4)	47 (71.2)	4 (18.2)	47 (72.3)	
No	14 (28.6)	19 (28.8)	18 (81.8)	18 (27.7)	
ECOG 0–1	38 (77.6)	44 (66.7)	5 (22.7)	49 (75.4)	
ECOG 2–4	11 (22.4)	22 (33.3)	17 (77.3)	16 (24.6)	
Bulky disease: Yes	18 (36.7)	4 (6.1)	0 (0)	21 (32.3)	
No	31 (63.3)	62 (93.9)	22 (100)	44 (67.7)	
BM infiltration: Yes	0 (0)	24 (63.6)	3 (13.6)	48 (73.8)	
No	49 (100)	42 (63.6)	19 (86.4)	17 (26.2)	
Prognostic scores	NCCN-IPI	NCCN-IPI	IELSG	FLIPI	
Low	14 (28.6)	5 (7.6)	7 (31. 8)	13 (20)	
Low-Intermediate	15 (30.6)	23 (34.8)			
Intermediate			8 (36.4)	23 (35.4)	
High-intermediate	14 (28.6)	28 (42.4)			
High	4 (8.2)	9 (13.6)	3 (13.6)	26 (40)	
N/A	2 (4.1)	1 (1.5)	4 (18.2)	3 (4.6)	
ALC > 1.1×10^9/l	34 (69.4)	43 (65.2)	14 (63.6)	ALC ≥ 1.1	46 (70.8)
ALC ≤ 1.1×10^9/l	13 (26.5)	18 (27.3)	5 (22.7)	ALC < 1.1	10 (15.2)
AMC < 0.62×10^9/l	27 (55.1)	30 (45.5)	9 (40.9)	AMC < 0.32	11 (16.9)
AMC ≥ 0.62×10^9/l	20 (40.8)	31 (47)	10 (45.4)	AMC ≥ 0.32	42 (69.2)
ALC/AMC > 2.6	25 (51.0)	38 (57.6)	10 (45.4)	ALC/AMC ≥ 4.7	27 (41.5)
ALC/AMC ≤ 2.6	22 (44.9)	23 (34.8)	9 (40.9)	ALC/AMC < 4.7	29 (44.6)
N/A	2 (4.1)	5 (7.6)	3 (13.6)		7 (10.8)
Cell of origin: GCB	23 (46.9)	20 (30.3)	2 (9.1)	Grade of FL	
Non-GCB	24 (49)	35 (53.0)	20 (90.9)	Gr I/II	45 (69.2)
N/A	2 (4.1)	11 (16.7)		Gr III	20 (30.8)
Therapy: IHT	49 (100)	66 (100)		65 (100)	
HD-MTX+CTR			10 (45.5)		
Therapy response: PR+CR	46 (93.9)	48 (71.7)	18 (81.8)	60 (92.7)	
PD+SD	3 (6.1)	18 (27.3)	4 (17.9)	5 (7.7)	
Disease relapse	17 (34.7)	13 (19.7)	9 (40.9)	27 (41.5)	
FL transformation to DLBCL				8 (12.3)	
Vital status: Alive	34 (69.4)	38 (57.6)	14 (63.6)	50 (76.9)	
Dead	15 (30.6)	28 (42.4)	8 (36.4)	15 (23.1)	

**DLBCL* Diffuse large B cell lymphoma, *Primary CNS DLBCL* Primary central nervous system DLBCL, *FL* Follicular lymphoma, *ECOG* Eastern Cooperative Oncology Group performance status, *BM* bone marrow, *ALC* absolute lymphocyte count, *AMC* absolute monocyte count, *GCB* germinal centre B cell, *IHT* immunochemotherapy, *HD-MTX* high doze methotrexate, *CTR* complete tumour resection, *CR* complete remission, *PR* partial remission

Immunohistochemistry

Immunohistochemical analysis was performed using a streptavidin-biotin complex technique and antibodies against the following antigens: CD10 (Novocastra), CD20 (DAKO), BCL2 (DAKO), BCL6 (DAKO), MUM1 (DAKO), CD44s (DAKO), CD34 (DAKO) and FOXP1 (Spring Bioscience). In order to determine the origin of cell (GCB vs. non-GCB type), Visco-Young algorithm (three-marker algorithm) was used [30]. The antibody against CD34 was used to stain

endothelial microvessel cells under light microscope. Immunohistochemistry was performed using a standard DAKO LSAB +/horseradish peroxidase technique. MVD was quantified in the three most vascularized areas ("hot spots") of the tumour in the light microscope at ×50 magnification and then each hot spot was counted at high power field using defined area of 0.36mm^{2}at ×400 magnification by three pathologists. The mean number of microvessels from three areas was used in further analyses. FL was characterized with significantly higher interfollicular than intrafollicular MVD which was consequently counted as result. Regarding CD44s antibody, the membranous staining in up to 10 % of malignant cells was considered as negative, 11–70 % of malignant cells was considered as positive, while over 70 % as strongly positive [31]. A uniform high nuclear expression of FOXP1 with cut-off 60 % and over, was used as FOXP1 positivity according to the Visco-Young algorithm [30].

Statistical Analysis

Survival analyses was done using the Kaplan-Meier method, while Fisher test and χ^2 test were used to analyse categorical variables. Receiver Operating Characteristic (ROC) curve was used to set the cut-off value of MVD for DLBCL, while median MVD value was set as cut off point for FL. The cut off points for ALC, AMC and ALC/AMC were used according to previously published data, including 1.1×10^9/L, 0.62×10^9/l and 2.6, respectively for DLBCL and 1.1×10^9/L, 0.32×10^9/l and 4.7, respectively for FL [11, 32, 33]. Spearman and Kendall's correlation coefficient were used to investigate correlation between MVD, and expression of CD44s with clinical parameters (clinical stage, National Comprehensive Cancer Network-IPI – NCCN-IPI, International Extranodal Lymphoma Study Group – IELSG score, Follicular Lymphoma International Prognostic Index – FLIPI, bulky disease, bone marrow involvement, performance status, therapy response, disease relapse, histological grade of FL, and higher grade of FL transformation). Mann-Whitney and Kruskal-Wallis tests were used to compare MVD, and expression of CD44s between different lymphoma subtypes. Multivariate Cox regression analysis was performed to select the most predictive variable for OS. All statistical tests were two-sided. P value ≤ 0.05 was considered as significant. All analyses were done in the SPSS program version 21.0 software (IBM SPSS).

Results

Overall response rate – ORR (complete remission–CR, partial remission–PR) was in DLBCL, primary CNS DLBCL, and FL achieved as following: 94/115 patients (81.7 %), 18/22 (81.8 %) and 60/65 (92.3 %), respectively. Disease relapse was confirmed in 26.1 % patients with DLBCL, 40.9 % with primary CNS DLBCL and 51.5 % with FL. Regarding ALC $> 1.1\times10^9$/l, AMC $< 0.62\times10^9$/l and ALC/AMC > 2.6, ORR was in DLBCL patients and primary CNS DLBCL: 82.4, 92.6, 86.7 %, respectively and 71.4, 62.5, 60 %, respectively, while regarding ALC $\leq 1.1\times10^9$/l, AMC $\geq 0.62\times10^9$/l, and ALC/AMC $\leq$ 2.6,ORR was 79.3, 70.8, 76.2 %, respectively and 100, 90, 100 %, respectively. ORR in FL patients with ALC $< 1.1\times10^9$/l, AMC $\geq 0.32\times10^9$/l, and ALC/AMC < 4.7 was achieved in 85.7, 95.2, 94 %, respectively, while in patients with ALC $\geq 1.1\times10^9$/l, AMC $< 0.32\times10^9$/l, and ALC/AMC $\geq$ 4.7, was 95.3, 87.5, and 95.8 %. In DLBCL and primary CNS DLBCL patients ALC, AMC did not correlate (ρ or $\tau > 0.3$) neither with clinical parameters nor with histopathological (cell of origin, MVD or CD44s expression), while regarding ALC/AMC there was observed that only ECOG PS 2–4 correlated with low ALC/AMC in DLBCL patients ($\tau = 0.467$, $p < 0.0001$) and in primary CNS DLBCL ($\tau = 0.478$, $p = 0.045$). In FL patients ALC, AMC and ALC/AMC did not correlate with any of analysed clinical or histopathological parameters.

According to Visco-Young algorithm, 14/44 GCB DLBCL cases had high FOXP1 tumour cell expression (33.3 %) and 44/59 non-GCB cases (74.5 %) ($\chi^2 = 15.42$, $p < 0.0001$). Furthermore, high FOXP1 expression was present in 8/23 (34.8 %) GCB nodal DLBCL and 15/24 (48.9 %) non-GCB cases ($\chi^2 = 3.61$, $p = 0.05$), while 6/19 (31.6 %) GCB extranodal DLBCL and 29/35 (82.9 %) of non GCB extranodal DLBCL ($\chi^2 = 14.19$, $p < 0.0001$) had high FOXP1 expression (Fig. 1). Only 4 cases of primary CNS DLBCL belonged to GCB type and 3 had low FOXP1 tumour cell expression (Fisher's Exact Test $p = 0.59$). Strong uniform expression of FOXP1 was found in 31 DLBCL (27.0 %) and 3 primary CNS DLBCL (13.6 %) [34].

Regarding MVD, there was difference among analysed subtypes of lymphoma ($p = 0.001$) with the highest MVD observed in interfollicular areas of FL, followed by extranodal DLBCL, nodal DLBCL and primary CNS DLBCL with median count of blood vessels of 45, 37, 32 and 26.5, respectively (Figs. 1 and 2). There was no statistical difference regarding MVD when compared nodal, extranodal DLBCL and primary CNS DLBCL ($p = 0.27$). Regarding CD44s expression, the lowest one was observed in FL, intermediate in nodal and extranodal DLBCL, while it was the highest in primary CNS DLBCL (30, 65, 80 and 85 % respectively) ($p = 0.021$) (Figs. 1 and 2).

The most discriminative cut-off value of MVD selected by the ROC analysis for DLBCL was 43 blood vessels (sensitivity 71.4 % and specificity 69.1 %) with an AUC value of 0.742 (95 % CI 0.618–0.866, $p = 0.01$). High MVD was observed in 44 DLBCL patients (38.3 %). Among analysed parameters, high MVD only correlated with primary resistant disease (PD, SD) ($\tau = 0.323$,

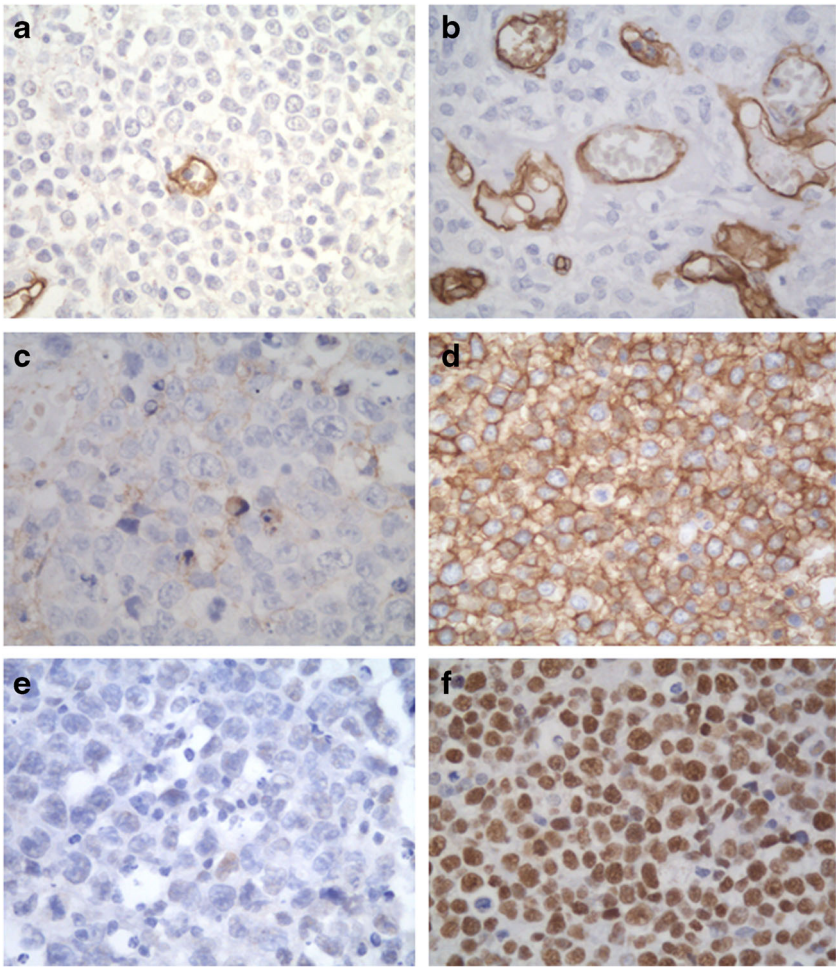

Fig. 1 CD34, CD44s and FOXP1 staining of DLBCL. Representative images of microvessel density (MVD) in tumour tissue highlighted by anti CD34 antibody in DLBCL (**a**) low MVD (original magnification ×400) and high MVD (**b**) (original magnification ×400). Low CD44s expression in DLBCL (**c**) (original magnification ×400) and high diffuse CD44s expression (**d**) (original magnification ×400). Low FOXP1 protein expression in biopsy samples of DLBCL (**e**) (original magnification ×400) and high FOXP1 tumour cell expression (**f**) (original magnification ×400)

$p < 0.0001$). High MVD was observed in 30 % primary CNS DLBCL patients and 52.3 % FL, but without any correlation with clinical or histopathological parameters. High CD44s expression was found in 53 % DLBCL patients, 60 % primary CNS DLBCL and 37.5 % FL, respectively (Figs. 1 and 2). Expression of CD44s was not in correlation with clinical or histopathological parameters.

Survival Analysis

There was no difference in survival between nodal and extranodal DLBCL (Log Rank = 1.96, $p = 0.162$). Moreover, in DLBCL patients there wasn't observed survival difference regarding gender and bulky disease. The presence of advanced Ann Arbor stage of lymphoma (III-IV) and B symptoms were of borderline significance on the OS (Log Rank = 8.05, $p = 0.045$; Log Rank = 3.88, $p = 0.049$, respectively). The NCCN-IPI was highly significant regarding OS (Log Rank = 24.45, $p < 0.0001$) and event-free survival (EFS) (Log Rank 22.45, $p < 0.0001$). ALC and AMC didn't affect neither EFS nor OS. However, ALC/AMC > 2.6 was associated with favourable EFS (Log Rank = 9.31, $p = 0.002$) and OS (Log Rank = 8.73, $p = 0.003$) (Fig. 3). Regarding cell of origin (GCB vs non-GCB), there was inferior OS (Log Rank = 6.56, $p = 0.009$), and EFS observed in non-GCB subtype of DLBCL (Log Rank = 7.53, $p = 0.006$). Furthermore, strong FOXP1 tumour cell expression in DLBCL affected both EFS and OS (Log Rank = 8.88, $p = 0.003$; Log Rank = 8.66, $p = 0.003$). High MVD correlated with poorer OS (median 35 months, Log Rank = 5.99, $p = 0.014$) and EFS (median 33 months, Log Rank = 6.06, $p = 0.014$) (Fig. 4). High CD44s expression did not affect survival ($p > 0.05$).

In primary CNS DLBCL patients the IELSG score was predictive for the OS in analysed group (Log Rank = 10.40, $p = 0.006$). ALC, AMC and ALC/AMC didn't affect survival, nor did high MVD or CD44s, but the patients who had complete tumour reduction previous to chemotherapy, had better OS (median not reached vs. 28 months, Log Rank = 4.73, $p = 0.03$).

Regarding FL, bulky disease, presence of B symptoms and Ann Arbor stage of lymphoma didn't have impact on the EFS and OS, while females experienced more aggressive disease

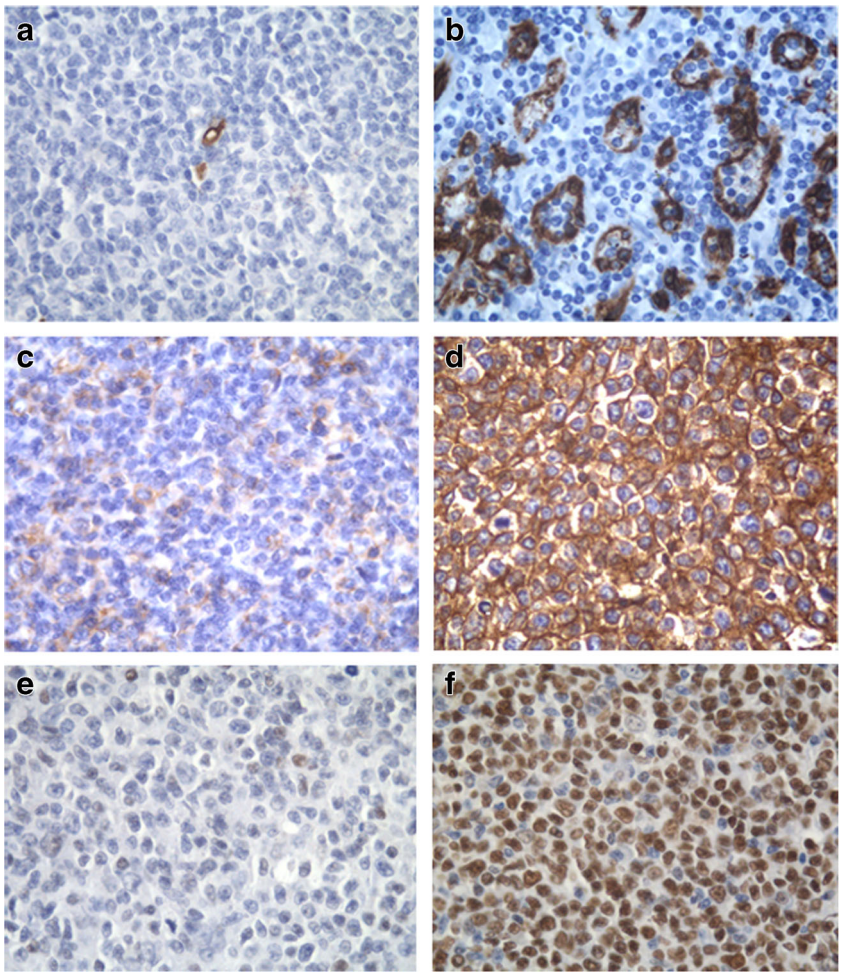

Fig. 2 CD34, CD44s and FOXP1 staining of FL. Representative images of microvessel density (MVD) of interfollicular area in tumour tissue highlighted by anti CD34 antibody (**a**) low MVD (original magnification ×400) and high MVD (**b**) (original magnification ×400). Low CD44s expression in FL (**c**) (original magnification ×400) and high diffuse CD44s expression (**d**) (original magnification ×400×400). Low FOXP1 protein expression in biopsy samples of FL (**e**) (original magnification ×400) and high FOXP1 tumour cell expression (**f**) (original magnification ×400)

(Log Rank = 4.93, $p = 0.026$). The FLIPI was highly significant for predicting OS (Log Rank = 15.13, $p = 0.001$) and EFS (Log Rank = 14.39, $p = 0.001$). The patients with ALC $\geq 1.1 \times 10^9$/l at diagnosis had superior EFS and OS (Log Rank = 16.25, $p = 0.0001$; Log Rank = 15.39, $p = 0.0001$) as well as with ALC/AMC ≥ 4.7 (Log Rank = 4.16, $p = 0.041$; Log Rank = 4.92, $p = 0.027$) compared to ALC $< 1.1 \times 10^9$/L and ALC/AMC < 4.7, while AMC level didn't have impact on the survival (Fig. 5). Patients with FL grade 3 had poorer OS than with grade 1 and 2 (60 months vs. median not reached, Log Rank = 7.34, $p = 0.007$) and EFS (45 months vs. median not reached, Log Rank = 7.21, $p = 0.007$). High MVD did not have impact on the OS ($p > 0.05$), while high CD44s expression correlated with inferior OS (51 months vs. median not reached, Log Rank = 8.75, $p = 0.003$) and EFS (45 months vs. median not reached, Log Rank = 7.67, $p = 0.006$) (Fig. 6).

Multivariate Cox regression analysis of DLBCL, among the most significant parameters in univariate analysis (NCCN-IPI, ALC/AMC, cell of origin according to the Visco-Young algorithm, high FOXP1 tumour cell expression and MVD) has shown that ALC/AMC was the most significant individual parameter that affected OS (HR 3.27, 95 % CI 1.51–7.09, $p = 0.003$) along with NCCN-IPI (HR 1.39, 95 % CI 1.08–1.79, $p = 0.01$), while other parameters lost its significance ($p > 0.05$). In FL, multivariate analysis revealed that among variables that were significant in univariate analysis (FLIPI, gender, ALC, ALC/AMC, advanced histological tumour grade, and CD44s expression), ALC/AMC was the most valuable parameter that influenced OS (HR 5.21, 95 % CI 1.17–23.21, $p = 0.03$), followed with the FLIPI (HR 3.98, 95 % CI 1.06–14.95, $p = 0.041$) while other parameters lost prognostic significance.

Discussion

Using GEP, two subtypes of DLBCL were defined including ABC and GCB [2]. Primary CNS DLBCL, according to the GEP, is characterized with specific signatures, greater heterogeneity and poorer outcome compared to nodal DLBCL, and is regarded as a separate entity due to a distinctive expression signature [2, 35]. On the contrary, FL is low grade lymphoma with mostly favourable

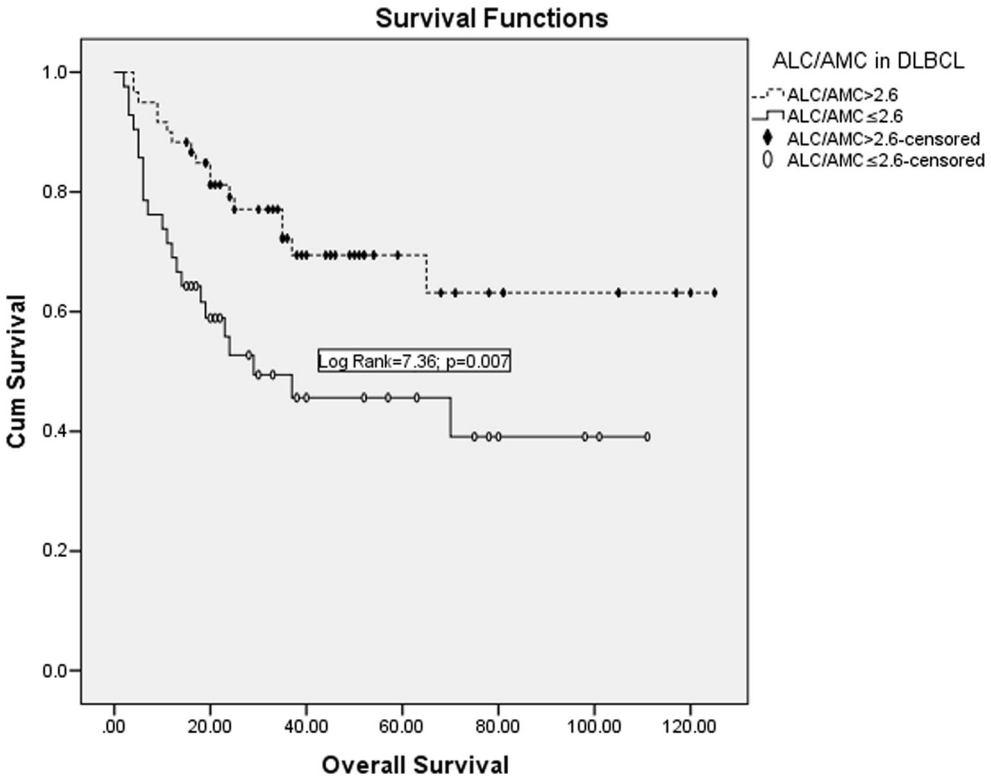

Fig. 3 Overall survival of DLBCL patients regarding absolute lymphocyte to monocyte ratio (ALC/AMC)

outcome, although up to 60 % of patients can transform into DLBCL [26]. Current available prognostic scores retained predictive value in rituximab era, which was confirmed in our study where the NCCN-IPI strongly influenced survival, while the IELSG and the FLIPI retained prognostic value for OS.

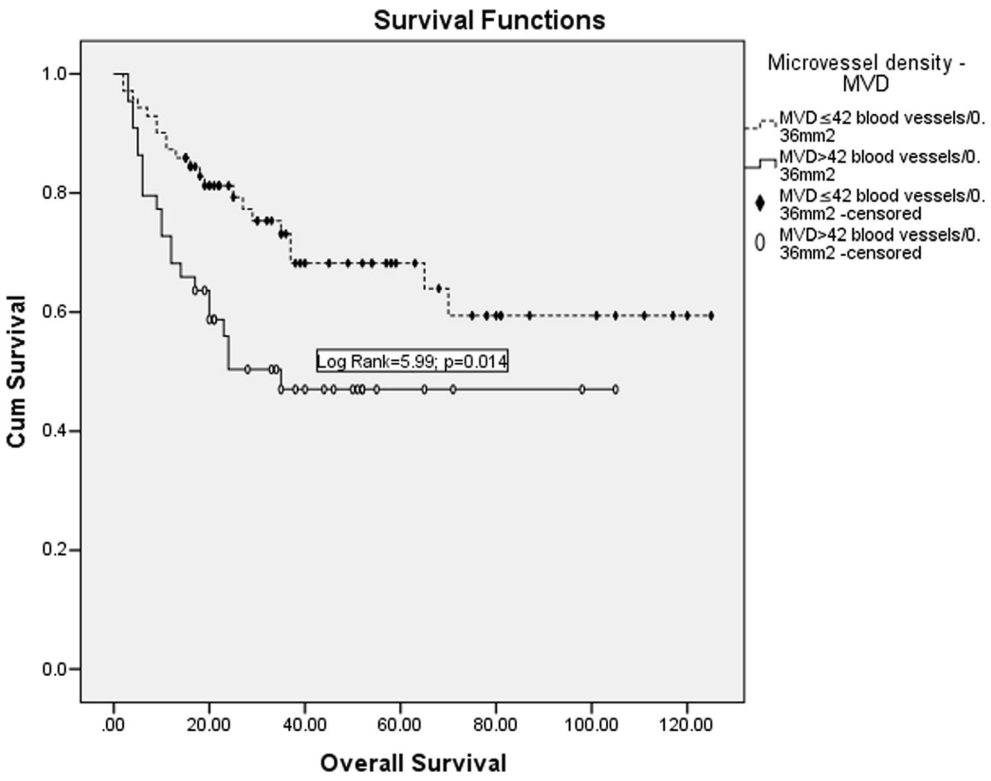

Fig. 4 Overall survival of DLBCL patients regarding microvessel density in tumour tissue-MVD

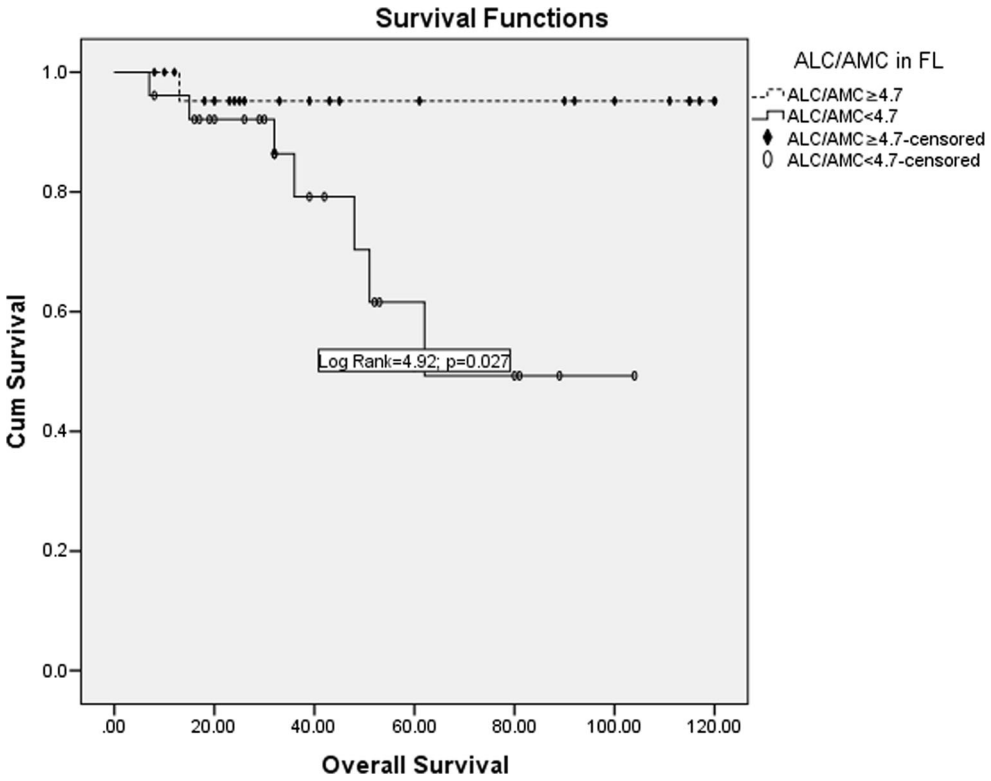

Fig. 5 Overall survival of FL patients regarding absolute lymphocyte to monocyte ratio (ALC/AMC)

The potential prognostic role of ALC, AMC and ALC/AMC, alone or in combination, as the biomarkers of immune microenvironment and host immunity was highlighted by the studies based on GEP and immunohistochemistry [4–11]. Our study didn't confirm the prognostic significance of ALC and AMC alone in DLBCL, while

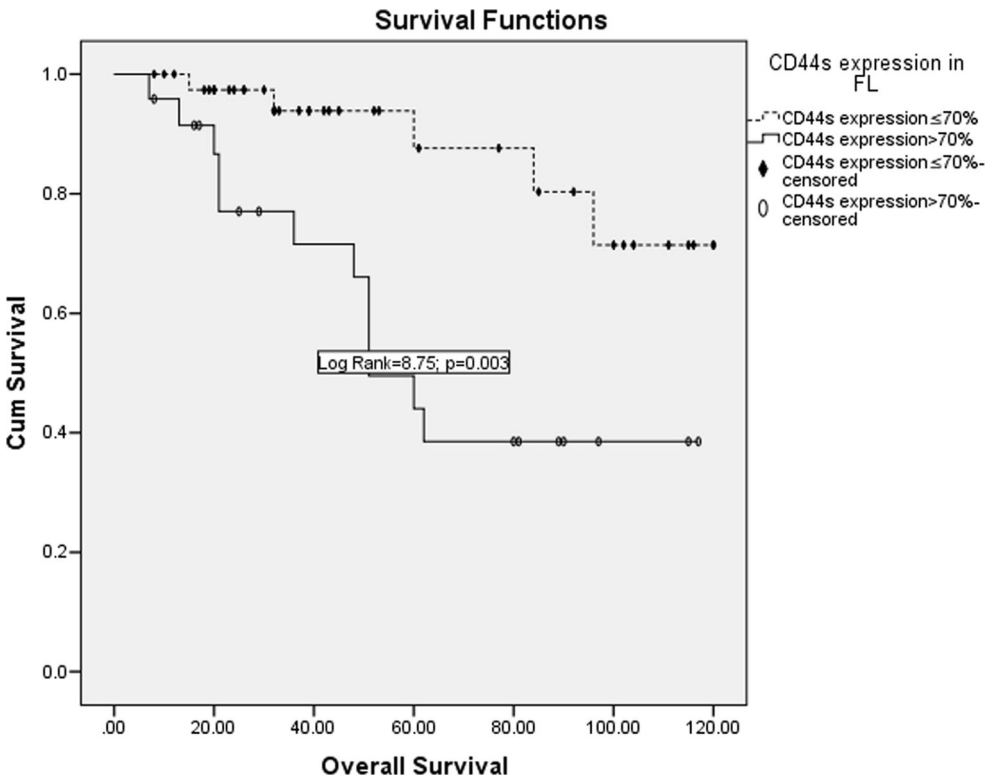

Fig. 6 Overall survival of FL patients regarding high CD44s expression

ALC/AMC in Cox regression model was highly prognostic (HR 3.80, 95 % CI 1.80–8.06). Just recently, the meta-analysis of Lin et al. which includes nine studies covering a total of 4198 subjects, showed that low ALC/AMC ratio at diagnosis do have an adverse effect on outcome for DLBCL patients treated with immunochemotherapy [36]. Regarding FL, the role of complete blood counton survival in FL patients treated with immunochemotherapy is less investigated and remains not well established [10, 11, 33]. ALC has been reported to correlate with treatment response and PFS, whose prognostic significance was confirmed in our study [37, 38]. However, we haven't found the prognostic role of AMC, which is supported by the study of Watanabe et al. despite that monocytosis was previously marked as possible negative predictive factor for outcome [10, 39, 40]. The role of ALC/AMC has to be more investigated, since some performed studies have reported different cut off points of ALC/AMC that were used to set the best value which influenced survival [11, 33]. We have used the cutoff point for ALC/AMC that was reported by Kumagai et al. in the study based on retrospective analysis of 99 patients with FL, and have found the significant influence of AMC/ALC on both EFS and OS, that were also highly prognostic when analysed in Cox regression model [11].

Angiogenesis is important for tumour growth, invasiveness and progression [41, 42]. The clinical significance of MVD is difficult to establish because heterogeneous studypopulations have been described, different treatments were applied and range of cell surface markers were used [21, 43]. Some studies have reported no correlation between MVD and treatment response in DLBCL patients treated with chemotherapy, however the degree of angiogenesis may be in correlation with disease status and vary between patients [44, 45]. GEP analyses, pointed out potential role of angiogenesis in DLBCL [14]. Cardesa-Salzmann et al. using CD31 antibody, have concluded that high MVD correlated with unfavourable prognosis independently of the IPI in patients treated with rituximab [14]. We have used anti-CD34 antibody which recognizes larger number of vessels, as previously reported, compared with anti-CD31 and anti-Factor VIII antibodies [41, 43]. Our data suggest inferior OS and EFS of DLBCL patients with high MVD, treated with immunochemotherapy, but not in primary CNS DLBCL whose outcome was probably altered by surgical approach. He et al. suggested that the presence of aggregative perivascular tumour cells (APVT) was associated with higher progression rates and inferior survival [17]. However, this field is modestly investigated and further studies are necessary. In FL, higher MVD is recognized in the interfollicular area compared to the follicles. Jørgensen et al. analysed pre-therapy FL cases and found that high interfollicular MVD correlated with higher incidence of disease progression, transformation to DLBCL, inferior EFS and OS [44, 46]. Since the historical group treated with CHOP protocol was analysed in this study, and our data do not support the significance of MVD on the outcome, this could be explained with the influence of rituximab based treatment, which altered the influence of MVD on OS in FL. Moreover, in order to improve survival in NHL, few studies, based on drugs with antiangiogenic action with promising results have been performed, but further investigations in this field are needed [47].

The CD44 transmembrane glycoprotein family and its associated proteins participate in the extracellular matrix changes that influence cell growth, survival and differentiation [22]. CD44s represents standard form of translated 10 of 20 exons of theCD44 gene, whose other 10 exons are spliced into variant isoforms (CD44v) which are mostly expressed on malignant cells [23, 24]. Previous studies have reported that CD44s and CD44 variants correlate with advanced stage of DLBCL and worst outcome [48]. As suggested, CD44s clustered with typical non-GCB DLBCL markers, which however is not confirmed in our study [31]. Furthermore, our data support the results of Wei et al. who have shown that the negative prognostic significance of CD44s decreased in rituximab treated patients [25]. He et al. reported lower survival rates in APVT with strong expression of CD44s in primary CNS DLBCL [17]. We haven't found the significance of CD44s expression in primary CNS DLBCL which might be result of surgical treatment approach that affected the outcome. On the other hand, the role of CD44s has been modestly investigated in low grade lymphomas. Higashi et al. proved that the expression of CD44s and CD44 variants were up regulated according to the diffuse evolution of FL [26]. Our study didn't confirm these results, however as an individual parameter high CD44s expression in tumour cells correlated with significantly poorer EFS and OS.

One of the limitations of our study is the retrospective nature and relatively limited sample size. However, we have tried, among cheap and available clinical and histopathological parameters, to emphasize the most important markers that might help to select the patients at higher risk of disease progression in order to consider therapy intensification.

In conclusion, the most important parameter that influenced survival in both DLBCL and FL was ALC/AMC along with NCCN-IPI or FLIPI. Furthermore, ALC/AMC at diagnosis is simple and robust tool that better defines clinical response and long-term survival of immunochemo therapy treated DLBCL and FL patients. Prospective studies are needed in order to incorporate ALC/AMC in

available prognostic indices for better distinction of patients at higher risk of a poor clinical outcome.

Compliance with Ethical Standards

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Conflicts of Interest The authors declare that they have no conflict of interest.

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