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ORIGINAL ARTICLE

The density of CD8+ T-cell infiltration and expression of BCL2 predicts outcome of primary diffuse large B-cell lymphoma of bone

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Abstract Primary bone lymphoma (PBL) comprises 5 % of all extranodal non-Hodgkin's lymphomas (NHLs). Diffuse large B-cell lymphoma (DLBCL) accounts for the majority of cases, which is the most heterogeneous group of lymphomas. Previous studies suggested that besides the tumor cell phenotype, phosphatidylinositol 3-kinase/acutely transforming retrovirus/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway activity and the composition of the immune-microenvironment of DLBCL influence the clinical behavior of the disease. The aim of our study was to determine the relationship between clinical factors, tumor cell phenotype, microenvironment, PI3K/AKT/mTOR pathway activity, and disease outcome in primary bone diffuse large B-cell lymphoma (PB-DLBCL). We constructed tissue-microarrays from 41 cases of PB-DLBCL. To characterize tumor cell phenotype, T-cell subsets, macrophages, and PI3K/AKT/mTOR pathway activity immunohistochemical stainings were evaluated. Kaplan–Meier survival analysis provided evidence that age (≤65), CD3 and CD8+ T cell infiltrations >5 %, low BCL2 expression of the tumor cells (≤30 %), and low proliferation index (Ki67≤57 %) were associated with favorable outcome of PB-DLBCL patients. Multivariate analysis revealed that CD8+ T cell infiltration >5 % and low BCL2 expression (≤30 %) were independent predictors of survival. Increased macrophage infiltration (>10 %) showed tendency toward an adverse prognostic effect. International prognostic index, tumor cell phenotype (GCB or ABC), MYC protein expression, and activation of PI3K/AKT/mTOR pathway had no significant impact on survival. However, mTOR activity showed a significant correlation with activated B-cell phenotype. We conclude that CD8 and BCL2 expressions are potential prognostic markers for PB-DLBCL patients and the PI3K/AKT/mTOR pathway appears to be an additional therapeutic target in PB-DLBCL with activated-B-cell phenotype.

Keywords Diffuse large B-cell lymphoma · Microenvironment · Primary bone lymphoma · Bone neoplasm

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Abbreviations

ABC	Activated B-cell
AKT	Acutely transforming retrovirus
BCL2	B-cell lymphoma 2
DLBCL	Diffuse large B-cell lymphoma
GCB	Germinal center B-cell
IPI	International prognostic index
PBL	Primary bone lymphoma
PB-DLBCL	Primary bone diffuse large B-cell lymphoma
PI3K	Phosphatidylinositol 3-kinase

mTOR	Mammalian target of rapamycin
MYC	Myelocytomatosis viral oncogene homolog
NHL	Non-Hodgkin's lymphoma
TMA	Tissue microarray
pS6	Phosphorylated ribosomal-S6 protein

Introduction

Diffuse large B-cell lymphoma (DLBCL) represents a heterogeneous group of high-grade B-cell lymphomas with considerable variations in survival, indicating marked intertumoral heterogeneity. This heterogeneity is reflected by the WHO subclassification of DLBCL to a limited extent, indicating that additional factors are relevant for disease outcome [1]. The international prognostic index (IPI) is, to date, the most successful clinical prognostic factor. However, cumulative evidence indicates that several biological factors are relevant predictors of survival, including tumor cell origin, derangements in signaling pathways regulating cellular growth and differentiation, and host immune response represented by the composition and functional status of the microenvironment [2]. It has been shown that the germinal center origin of the tumor cells, increased T cell infiltration, low expression of tumor-infiltrating macrophages, and recently described low expression of MYC protein and low activity of the PI3K/AKT/mTOR signal transduction pathway are predictors of favorable survival for DLBCL patients [3–6].

Primary bone DLBCL has been defined as a distinct clinicopathological entity comprising less than 5 % of all extranodal lymphomas [7]. Nodal and extranodal DLBCLs share many pathological and clinical features. However, PB-DLBCL is considered as a lymphoma with a more favorable prognosis compared to nodal DLBCL [8–10]. The limited number of studies available on PB-DLBCL showed that younger age, early stages of the disease, low IPI scores, rituximab+cyclophosphamid+doxorubicin+vincristin+prednisone (R-CHOP) treatment, centroblastic morphology, and germinal center origin of the tumor cells are associated with a more favorable outcome [8–15].

The aim of the present study is to identify relevant biomarkers in addition to the main clinical prognostic factors which may determine disease outcome. We analyzed the composition of the tumor microenvironment, tumor cell phenotype, proliferation, and activity of the PI3K/AKT/mTOR signaling pathway in 41 patients with PB-DLBCL by immunohistochemical analysis using tissue microarray. Our results indicate that a higher number of tumor-infiltrating CD8+ T cells and lower BCL2 expression of the tumor cells represent independent favorable prognostic markers of survival in PB-DLBCL. The activation of the mTOR pathway is associated with activated B-cell phenotype of the tumor cells. However, it is not associated with patient survival.

Materials and methods

Patient selection

Forty-one patients with PB-DLBCL were selected from the Semmelweis University ($n=18$) and the Leiden University Medical Center ($n=23$). This patient cohort is a subgroup of a larger cohort recently published by Koens at al [16]. PB-DLBCL was defined as a monostotic disease with or without regional lymph node involvement or polyostotic disease affecting multiple skeletal sites without extraosseus organ involvement [7]. Disease staging was based on a combination of conventional and CT scan imaging, bone scanning, and bone marrow biopsy. Monostotic disease was scored as stage I, monostotic disease with adjacent soft tissue and regional lymph node involvement were staged as II, and multifocal bone involvement was scored as stage IV. The histological diagnosis of DLBCL was confirmed by two hematopathologists according to the WHO classification [1].

Tissue microarrays

Tissue microarrays (TMA) were constructed with a computer-driven semi-automated instrument (TMA Master, 3D HISTECH Ltd., Budapest, Hungary) by selecting representative tumor areas based on hematoxylin–eosin stained slides. Triplicate cores of 1 and 2 mm diameter were arrayed from the tumor samples into the recipient blocks.

Immunohistochemical analysis

TMA blocks were cut to 3-μm sections and applied to silane-coated adhesive microscope slides. Immunohistochemical staining was performed on dewaxed sections following antigen retrieval at 100 °C for 40 min in an electric cooker using a buffer of Tris-EDTA (pH 9.0) or Citrate (pH 6.0) or Target Retrieval Solution (Dako Corporation, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked with hydrogen peroxide pre-treatment for 20 min. The sources of antibody clones used for immunophenotyping are listed in Table 1. Sections were stained using a biotin-free anti-rabbit/mouse IgG polymer-peroxidase conjugate system (Novolink, Leica, Newcastle, UK). Immunoreactions were revealed using a diaminobenzidine (DAB) chromogen-hydrogen peroxide substrate for 5 min using the above kit. Harris hematoxylin was applied for highlighting the cell nuclei. Appropriate internal and external controls were used as positive and negative controls. Immunostained TMA slides were scanned using a Panoramic scan instrument (3D HISTECH) equipped with a×

Table 1 Antibodies used for immunohistochemistry

Primary antibody	Clone	Manufacturer
GCET1	RAM341	Abcam, Cambridge, UK
BCL6	PG-B6p	Dako, Glostrup, Denmark
CD10	56C6	Novocastra, Leic a Biosystems, UK
FOXP1	Jc12	Abcam, Cambridge, UK
MUM1	MUM1p	Novocastra, Leica Biosystems, UK
LMO2	1A9-3D11	Abcam, Cambridge, UK
CD21	1 F8	Dako, Glostrup, Denmark
Ki67	MIB-1	Dako, Glostrup, Denmark
CD3	F7.2.38	Dako, Glostrup, Denmark
CD4	4B12	Novocastra, Leica Biosystems, UK
CD8	4B11	Novocastra, Leica Biosystems, UK
CD57	NK1	Novocastra, Leica Biosystems, UK
CD68	KP1	Dako, Glostrup, Denmark
FOXP3	Poly.	Novus Biologicals, Littleton, USA
TIA	TIA-1	Abcam, Cambridge, UK
Granzyme B	GrB-7	Dako, Glostrup, Denmark
PS6	P-S6 RS6P	Cell Signaling, USA
BCL2	124	Dako, Glostrup, Denmark
MYC	Y96	Abcam, Cambridge, UK

20 Carl Zeiss objective (NA=0.83; Carl Zeiss MicroImaging Inc., Jena, Germany).

The relative proportion of positive cells for the different markers were determined on the scanned pictures at×400 magnification by counting 200 cells in three different areas of each TMA core sample by two independent investigators; a minimum of 0.1-mm^2 area was evaluated. Ordinary tissue sections were also stained in some cases, and the results were compared with the TMA results. The staining intensity was considered only for detecting phosphorylated ribosomal-S6 protein (pS6). Cytoplasmic reaction of the plasma cells with pS6 antibody was scored 3+ and used as positive internal controls, while the maximum intensity of the reaction in the reactive lymphocytes was scored 1+. PS6 staining was considered positive in cases where the tumor cells showed moderate (2+) or strong (3+) cytoplasmic reaction and the percentage of the positive tumor cells were above 10 %. The expression levels of MYC (nuclear) and BCL2 (cytoplasmic) proteins were assigned in the following categories: negative< 10 %, 10–30 % (+low), 30–50 % (++intermediate), 50–80 % (+++ high), and >80 % (++++very high). Here, 10 and 30 % cut-off values were used for positivity of MYC based on previous studies [6,17]. For BCL2, results were published with 30 and 50 % cut-offs [6,14–17]. Both values resulted to the same distribution in our PB-DLBCL cohort as no case showed expression between 30 and 50 %. Tumors were classified as germinal center B-cell (GCB) or activated B-cell (ABC) immunophenotype based on the expression of GCET1, CD10, MUM1, FOXP1, and LMO2 according to Tally's algorithm [18]. The tumor cell phenotype was also determined by Hans' algorithm based on the expression of CD10, BCL6, and MUM1 [19]. All tissue samples were handled in a coded fashion, according to the Dutch and Hungarian National Ethical guidelines (National Ethical Review Board approval: TUKEB no. 7/2006).

Statistical analysis

The association between the different clinicopathological and immune parameters was estimated and compared using the Mann–Whitney U-test for continuous variables. Categorical data were compared using Fisher's exact test or Pearson chi-square statistics. Bonferroni correction was not performed. A p value of <0.05 was considered as statistically significant. The Kaplan–Meier method was used for the comparisons of overall survival. Variables showing $p<0.05$ in univariate analysis (age, CD3, CD8, BCL2, and Ki67) were included in the Cox regression model for multivariate analysis. All statistical analyses were performed using the SPSS 13.0 software.

Results

Patients' characteristics, clinical data

A cohort of 41 patients with PB-DLBCL was studied. The clinical characteristics of the patients, including gender, age, stage, tumor localization, IPI scores, treatment, and response, are summarized in Table 2. The IPI score (composed of age, performance status, serum lactate dehydrogenase level, stage, and number of extranodal disease sites) was available for 31/41 cases. Twenty-four patients had an IPI score ≤2 (77 %); the mean score was 1.8. Ninety-four percent of the patients received combination therapy in the form of surgical resection, polychemotherapy (CHOP, $n=18$; rituximab–CHOP, $n=12$; CHOP-like, $n=4$, polychemotherapy unknown, $n=4$), and radiotherapy; two patients were treated with surgery and radiotherapy; and one patient did not get any treatment. For the 41 PB-DLBCL patients, the overall survival (OS) at 5 and 10 years was 78.5 %, with a median follow up of 36 months. The overall response rate and complete remission rate were 90.2 % (37/41) and 78.5 % (32/41), respectively. Two patients out of those in complete remission had multiple relapses treated with radiotherapy and polychemotherapy and no evidence of disease at 60 and 144 months. Nine patients (21.5 %) died within 24 months after diagnosis with partial response (5 cases) or progressive disease (4 cases). No significant difference of outcome was observed in our small cohort of patients treated with CHOP or CHOP-like chemotherapy ($n=22$; 10-year OS, 81.8 %) compared to patients treated with R-CHOP ($n=12$; OS, 83.3 %).

Table 2 Clinical parameters of patients with primary bone-diffuse large B-cell lymphoma

	Response to treatment		Total
	CR ($n=32$)	PR/PD ($n=9$)	($n=41$)
Gender	9/23	3/6	12/29
F/M	45 (11–78)	70 (43–78)	50 (11–78)
Age			
Median (range)			
Stage			
Stage I	22	6	28
Stage II	3	1	4
Stage IV	7	2	9
Location			
Femur	11	4	15
Other long bones	6	1	7
Hip	4	1	5
Other sites	4	1	5
Multiplex	7	2	9
IPI			
0–2	19	5	24
3–5	4	3	7
Unknown	9	1	10
Treatment			
CHOP/CHOP-like/PU	20	6	26
R-CHOP	10	2	12
No chemotherapy	2	1	3

CHOP cyclophosphamide, doxorubicin, vincristin, prednisone; *CHOP-like* cyclophosphamide, teniposide, doxorubicin, vincristin, bleomycin, prednisone; *CR* complete remission; *F* female; *IPI* international prognostic index; *M* male; *PU* polychemotherapy unknown; *PD* progressive disease; *PR* partial remission; *R-CHOP* rituximab–CHOP

Impact of clinical prognostic factors on survival

Age above 65 years was an unfavorable prognostic factor: 10-year OS for patients age ≤65 ($n=31$) and >65 years ($n=10$) were 86.4 and 50.0 %, respectively ($p=0.008$). Survival of patients with low (≤2) IPI scores was more favorable than of those with high (>2) IPI scores. However, the difference was not significant (80.8 and 57.1 %, $p=0.189$). Stage showed no significant impact on outcome; the 10-year OS with localized (stage I) and with advanced stage (stage II and IV) disease were 80.0 and 78.6 %, respectively. The results of the univariate analysis are shown in Table 3.

Impact of tumor cell phenotype on survival

Tumor cell phenotype was determined by Tally's [18] and Hans' [19] algorithms. The results showed 95 % concordance: 2 cases with activated B-cell (ABC) phenotype (CD10-, Bcl6-MUM1-) determined by Hans' algorithm were germinal center B-cell like (GCB) with Tally's phenotyping (FOXP1+, CGET1+, LMO2+). Applying the Tally's algorithm, we classified 22 cases as GCB and 19 cases as ABC-type. No significant difference of age was observed between the two groups ($p=0.681$). Patients with GCB-type tumors had more favorable prognosis (10-year OS for GCB-type tumors, 85.7 %) than ABC-type (10-year OS for ABC-type tumors, 66.8 %). However, the association between tumor cell phenotype and survival was not significant ($p=0.193$). MYC protein expression above 10 % was detected in 16/40 cases, including cases showing low (10–30 %, $n=6$), intermediate (30–50 %, $n=5$), and high expression (50–80 %, $n=5$), both in ABC and GCB subtypes (Fig. 1a–c). No correlation between MYC expression and proliferation rate assessed by Ki67 was observed ($p=0.65$). Kaplan–Meier analysis showed no significant difference in 10-year OS of patients with or without MYC protein expression using 10 or 30 % cut-off for positivity ($p=0.664$ and $p=0.599$, respectively; Table 3). BCL2 protein (>50 %) was detected in 11/39 cases with high (50–80 %, $n=5$) and very high (80–100 %, $n=6$) expressions (Fig. 1d–f). BCL2 expression was more common in the ABC subtype ($p=0.037$) and was associated with inferior OS ($p=0.001$; see Fig. 2a). Stratifying the patients according to tumor cell type (ABC/GCB), BCL2 expression remained an adverse prognostic factor in both subgroups (ABC, $p=0.019$; GCB, $p=0.003$). Concurrent expression of BCL2 and MYC had no further effect on OS compared to BCL2 expression alone (Fig. 2b).

Impact of the tumor microenvironment on survival

The tumor microenvironment was characterized by the frequencies of CD3, CD4, CD8, FOXP3, CD57 positive T-cells, Granzyme B and TIA expressing T and NK-cells, and CD68+ macrophages. A significant correlation between tumor-infiltrating T-lymphocytes represented by CD3 and CD8 expression and OS was found: the 10-year OS was 88.7 and 91.5 % for patients with high intensity and 47.6 and 55.0 % with low intensity of CD3+ and CD8+ T-cell infiltration, respectively ($p=0.004$ and $p=0.006$) (Fig. 2c, d; Table 3). The cut-off value for positivity was set at 5 %. High proliferation rate (Ki67>57 %) was more common in cases with low (≤5 %) CD3 and CD8 (4/12 and 4/16) compared to high (>5 %) CD3 and CD8 T cell infiltration (4/27 and 4/24), but the association was not significant ($p=0.3884$ and $p=0.6853$). TMA spots of two representative cases with low T cell infiltration and high Ki67 expression vs. high T cell infiltration and low Ki67 expression are shown in Fig. 3a, b. Calculated 10-year OS was 87.1 % for patients with low infiltration of CD68+ macrophages (cut-off value was 10 %) compared to those with high CD68 cell infiltration (60.0 %, $p=0.058$). Other investigated immune cells of the

Table 3 Univariate analyses for overall survival in patients with primary diffuse large B-cell lymphoma of bone

Parameter	Subgroup 1			Subgroup 2			*p*
	Cut-off	*n*	10y-OS (%)	Cut-off	*n*	10y-OS (%)	
Age	≤65 years	31	86.4±6.3	>65 years	10	50.0±15.8*	**0.008**
IPI	≤2	24	80.8±8.3	>2	7	57.1±18.7	0.189
Stage	≤1	28	80.0±7.9	>1	13	78.6±12.1	0.869
Cell of origin	ABC	19	66.8±11.2	GCB	22	85.7±7.6	0.193
Ki67	≤57 %	26	84.4±7.2	>57 %	8	31.3±17.8*	**0.007**
CD3	≤5 %	12	47.6±15.0	>5 %	27	88.7±6.1	**0.004**
CD4	≤5 %	20	64.0±10.9	>5 %	20	90.0±6.7	0.061
CD57	≤1.8 %	19	67.5±11.0	>1.8 %	19	89.5±7.0	0.072
FOXP3	≤0.9 %	19	68.4±10.7	>0.9 %	20	82.5±9.2	0.320
CD8	≤5 %	16	55.0±12.7	>5 %	24	91.5±5.8	**0.006**
TIA	≤2.2 %	20	74.3±10.0	>2.2 %	20	80.0±8.9	0.646
GRB	≤0.4 %	18	72.2±10.6	>0.4 %	22	81.3±8.5	0.526
CD68	≤10 %	24	87.1±7.0	>10 %	14	60.0±13.2	0.058
pS6	≤10 %	20	84.4±8.3	>10 %	21	71.1±10.0	0.300
MYC	≤30 %	30	79.4±7.5	>30 %	10	70.0±14.5	0.599
BCL2	≤30 %	28	92.9±4.9	>30 %	11	31.8±14.9*	**0.001**

Significant *p* values are marked with bold. *48-month overall survival values are presented instead of 10-year overall survival, while follow up times do not reach 120 month in the subgroup. *N*<41 for the following markers: IPI, *n*=31; Ki67, *n*=34; CD57; CD68, *n*=38; CD3; FOXP3; BCL2, *n*=39; CD8; CD4; TIA; GRB; MYC, *n*=40

10y-OS 10-year overall survival, *ABC* activated B-cell, *BCL2* B-cell lymphoma 2, *GCB* germinal center B-cell, *GRB* Granzyme B, *IPI* international prognostic index, *pS6* phosphorylated ribosomal-S6 protein

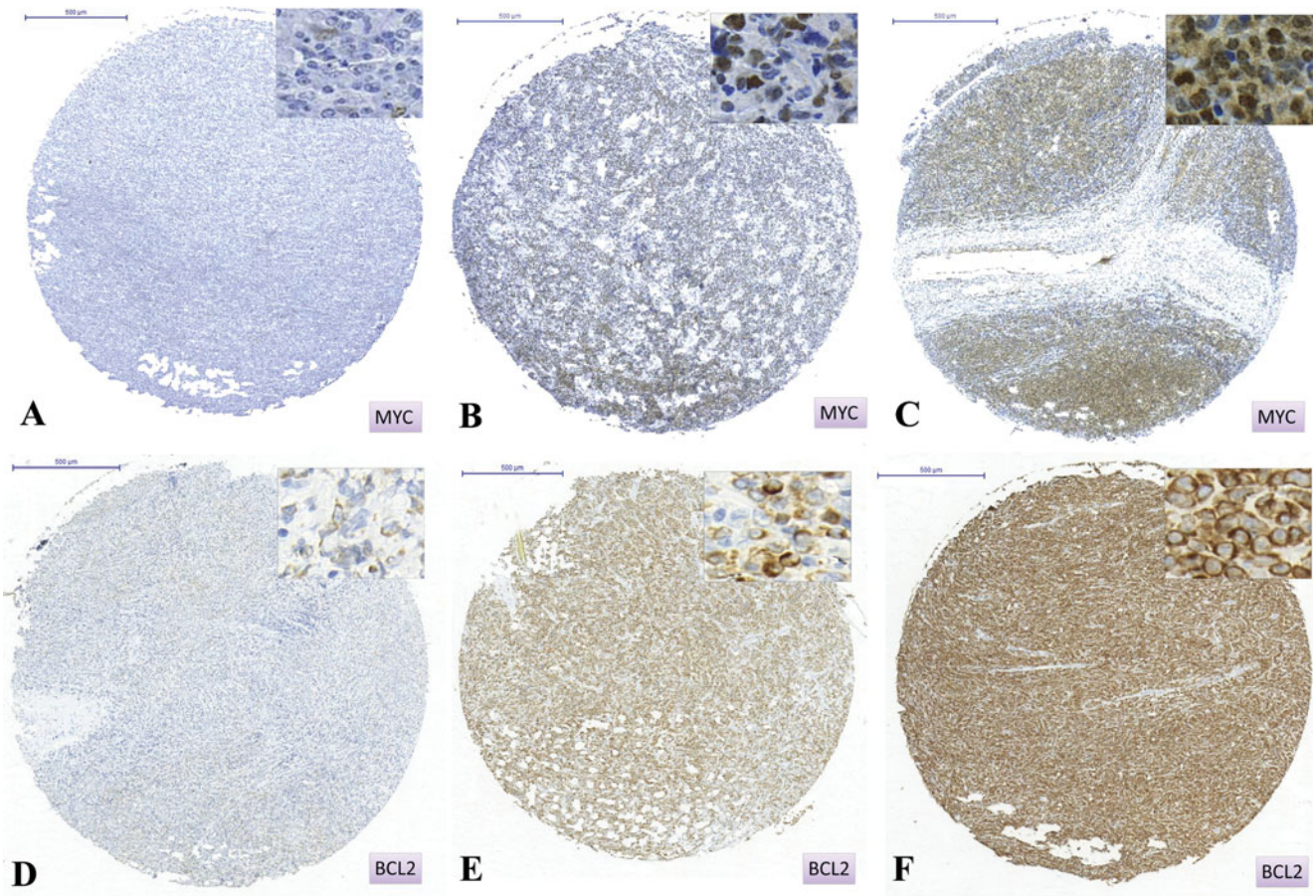

Fig. 1 Representative immunohistochemical (IH) tissue microarray cores from samples of patients with primary bone diffuse large B-cell lymphoma. IH analysis of MYC: **a** MYC<10 %, considered negative, **b** intermediate expression (30 %<MYC<50 %), and **c** high expression (50 %<MYC<80 %). IH analysis of BCL2: **d** BCL2≤30 %, considered negative, **e** high expression (50 %<BCL2<80 %), and **f** very high expression (BCL2>80 %)

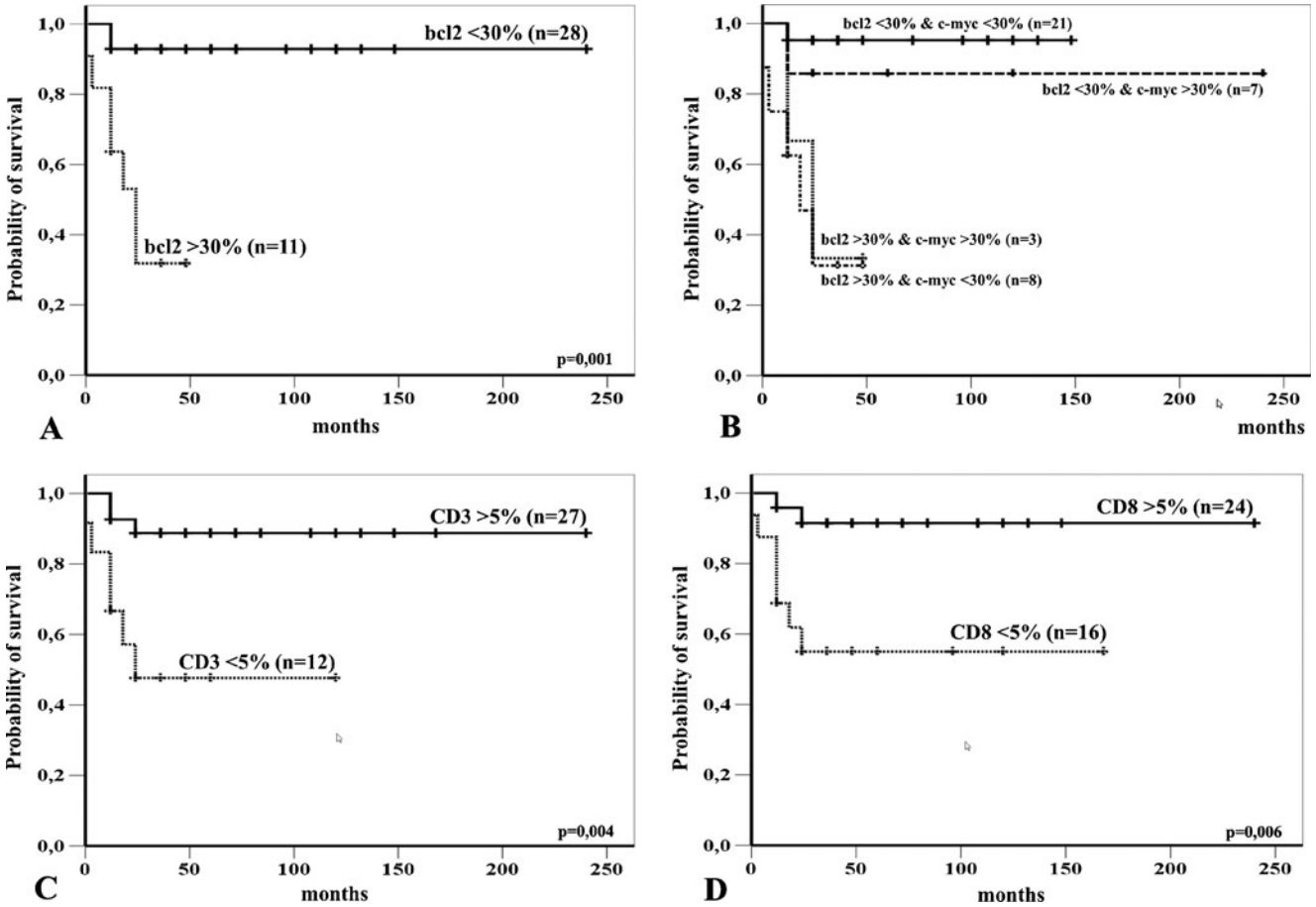

Fig. 2 Kaplan–Meier OS analysis in primary bone-diffuse large B-cell lymphoma with respect to the phenotypic characteristics. Analysis for T cell markers (CD3, CD8) were performed with evaluable immunostainings (CD3, BCL2; $n=39$, CD8, MYC; $n=40$). **a** Comparison of OS of patients with BCL2 expression≤30 % and >30 %, **b** comparison of overall survival of patients stratified according to BCL2 and MYC protein expression, **c** comparison of OS of patients with CD3+ T-cell infiltration ≤5 and >5 %, **d** comparison of OS of patients with CD8+ T-cell infiltration ≤5 and >5 %. Statistical significances are shown for BCL2, $p=0.001$; CD3, $p=0.004$; CD8, $p=0.006$, while for MYC, no significant association was found

microenvironment, FOXP3 expressing regulatory T lymphocytes (T-regs), follicular (CD57), and cytotoxic granule containing cells (TIA, Granzyme B) showed significantly lower intensity of infiltration compared to the major T cell populations (CD3, CD4, and CD8) and no correlation with OS. Table 3 includes the results of the univariate analysis regarding these T cell subgroups using the median value as a cut-off.

Impact of tumor cell proliferation rate on survival

Tumor cell proliferation was assessed by Ki67 expression. The proliferation rate exceeded 20 % in all cases (Ki67 median, 42.8; range, 24–90). Survival analysis (Table 3) indicated a favorable outcome for cases with low proliferation rate with a cut-off at 57 % ($p=0.007$). High Ki67 was more common in BCL2 positive (>30 %, 4/11) compared to BCL2 negative (≤30 %) cases (4/21); however, the association was not significant ($p=0.40$).

Activity of the PI3K/AKT/mTOR signal transduction pathway correlates with tumor cell phenotype

mTOR activity was detectable in 21/41 cases (51 %) by positive immunostaining for pS6 protein as the most sensitive marker of mTOR activity as shown by Egerváry et al. [20]. A significant difference was observed between GCB and ABC-type tumors as shown in Fig. 4a: pS6 protein staining was considered positive in 68 % of the tumors with ABC versus 36 % with GCB phenotype ($p=0.04$). Representative TMA array spots with IH detection of pS6 are shown in Fig. 4b, c, demonstrating pS6 protein expression in 3 % of tumor cells of a GCB-derived and 65 % of the tumor cells of an ABC-derived PB-DLBCL. Treatment response and disease outcome showed no correlation with mTOR activity: pS6 protein was detected in 15/32 (46.9 %) patients with CR and 6/9 (66.7 %) patients with PR or PD. Ten-year OS for patients without ($n=20$) and with mTOR activity ($n=21$) was 84.4 and 71.1 %, respectively ($p=0.3$; Table 3).

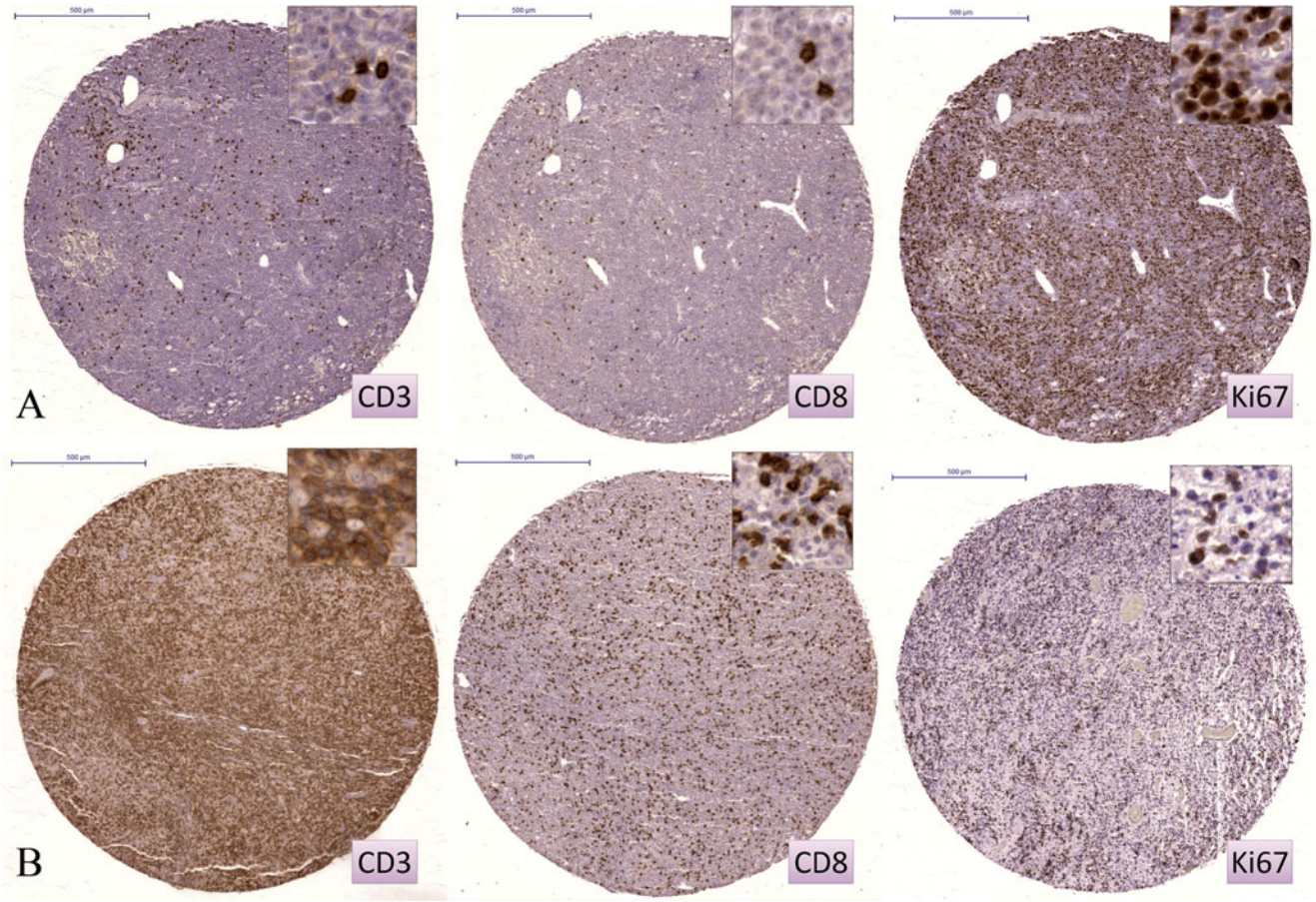

Fig. 3 Representative immunohistochemical (IH) tissue microarray cores from samples of patients with primary bone diffuse large B-cell lymphoma. **a** CD3, CD8, and Ki67 IH analysis of a patient with progressive disease, who died 24 months after diagnosis with low CD3 (3.1 %) and CD8 (1.7 %) T-cell infiltrations and 56 % proliferation rate detected by Ki67 positivity. **b** CD3, CD8, and Ki67 IH analysis of a patient in complete remission showing high CD3+ (58 %) and CD8+ (14 %) T-cell infiltrations and 28 % proliferation rate detected by Ki67 positivity

Multivariate analysis

Multivariate analysis of all 41 patients provided evidence that BCL2 expression (HR, 8.62; 95 % CI, 1.01–73.8; $p=0.049$) and intensity of CD8+ T-cell infiltration (HR, 23.1; 95 % CI, 1.18–454.1; $p=0.039$), but not CD3+ T-cell infiltration (HR, 14.6; 95 % CI, 0.48–203.6; $p=0.138$) and Ki67 proliferation index (HR, 4.19; 95 % CI, 0.85–20.7; $p=0.079$), are independent predictors of survival (Table 4).

Discussion

Several efforts have been made to determine prognostic markers for the survival of patients with nodal or extranodal DLBCLs including PB-DLBCL. In PB-DLBCL, clinical prognostic factors, IPI score, IPI risk factors, disease stage, and patient age were shown to be the most relevant predictors of survival [10–12]. Immunohistochemical studies are limited and show controversial results regarding the effect of tumor cell phenotype on the prognosis. To our knowledge, no published data are available on the prognostic effect of the immune response regarding PB-DLBCL.

To identify subgroups of patients at high risk of treatment failure, we analyzed tumor cell and microenvironment-related biomarkers on 41 cases of PB-DLBCL. Tumor-infiltrating CD8+ T cell density and BCL2 expression of the tumor cells showed the strongest correlation with disease outcome. Our study also confirmed that cytotoxic T-cell infiltration and BCL2 expression are independent prognostic variables.

Prognostic significance of the host response represented by the T-cell infiltration has been described by several studies in DLBCL [4,21,22]. The prognostic significance of CD8+ T cell infiltration found in PB-DLBCL is highly comparable to those demonstrated in nodal DLBCL [23,24]. These data suggest that a sufficient anti-tumor immune response improves the therapeutic effect and survival of patients with DLBCL. Furthermore, it supports the hypothesis that loss of tumor immunosurveillance results in a poor therapeutic response and outcome. Several lines of evidence suggest that low T-cell response is related to loss of expression of major histocompatibility complex (MHC) molecules on the tumor

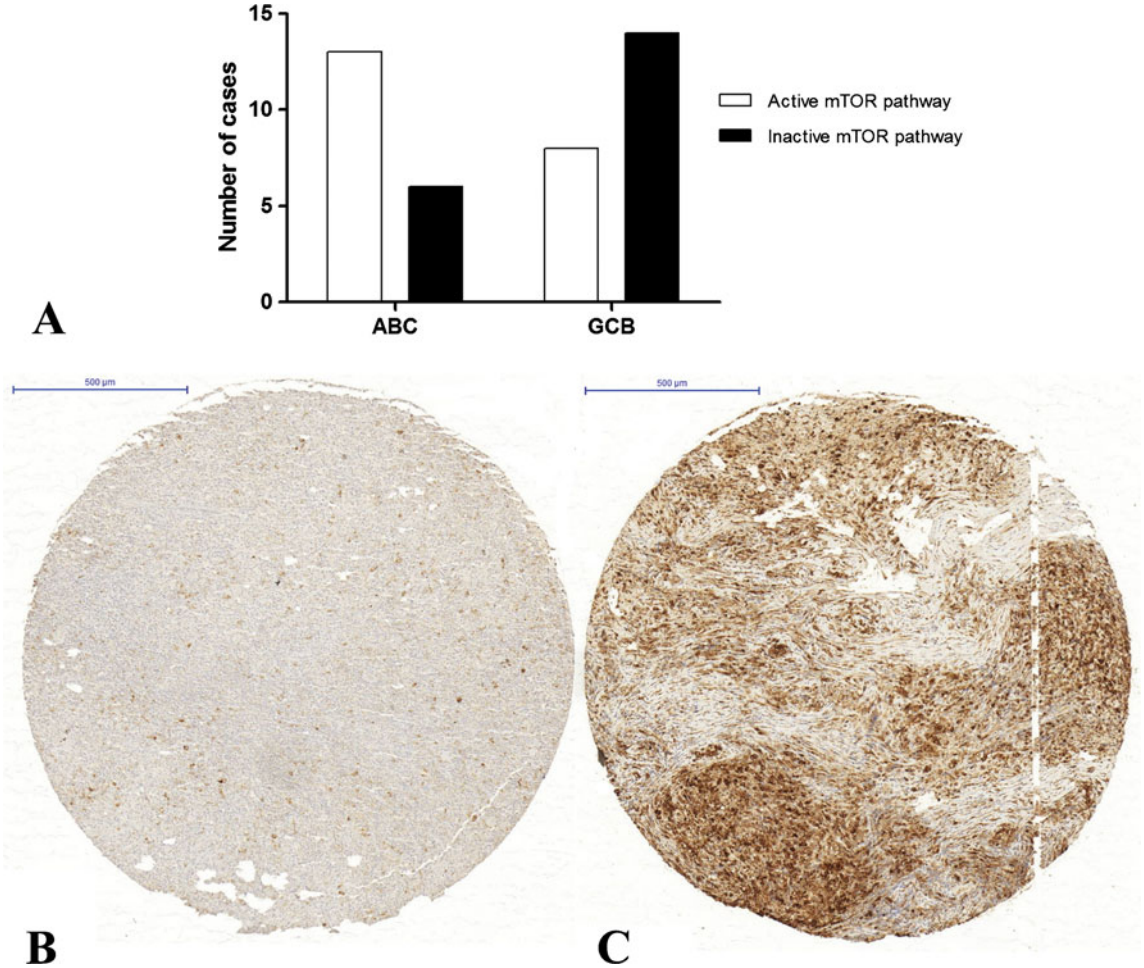

Fig. 4 **a** Column chart showing distribution of primary bone diffuse large B-cell lymphomas with active or inactive mTOR pathway regarding the tumor cell phenotype (activated and germinal center B-cell phenotype). Activated B-cell phenotype shows significant correlation with activated mTOR pathway ($p=0.04$). Representative tissue microarray cores of phospho-S6 IH analysis: **b** a representative PB-DLBCL case with germinal center B-cell phenotype and negative mTOR activity since <10 % of the tumor cell show positive reaction for pS6 protein (cut-off for pS6 positivity was set at 10 %), **c** a representative PB-DLBCL case with activated B-cell phenotype with profound mTOR activity since 65 % of the tumor cells showed positive reaction for pS6

cells, representing an important mechanism helping the lymphoma cells with B-cell origin to escape host immune defense mechanisms [4,24].

Regulatory T-cells, a subpopulation of CD4+ T-cells, are also capable of suppressing the activation of other immune cells, and recently, much attention has been devoted to this T-cell subpopulation as a prognostic factor in different B-cell lymphomas [25,26]. Based on our results, the number of tumor-infiltrating FOXP3+ regulatory T cells (Tregs) in PB-DLBCL showed no association with disease outcome, and it may not represent a relevant prognostic factor for this type of lymphoma.

TIA and Granzyme B cytotoxic granules are expressed in NK-cells and cytotoxic T cells, which play also an important part in anti-tumor immunity. However, only the negative influence of these cells has been found by Muris et al. describing a correlation between dense infiltration of TIA and Granzyme B+T-cells and poor survival in nodal DLBCL [27]. This result suggests that the cytotoxic immune response can be inefficient or even harmful in some situations. In our study, a similar correlation was not detected. Our different observation may reflect the distinct biology of PB-DLBCL or related to the relatively low number of cases.

Table 4 Multivariate analysis for overall survival in patients with primary diffuse large B-cell lymphoma of bone

Parameter	Risk group	*p*	HR	95 % CI
Age	>65 year	0.169	2.86	0.64–12.7
Ki67	>57 %	0.079	4.19	0.85–20.7
CD3	≤5 %	0.138	9.86	0.48–203.6
CD8	≤5 %	**0.039**	23.1	1.18–454.1
BCL2	>50 %	**0.049**	8.62	1.01–73.8

Significant *p* values are marked with bold

HR hazard ratio, *OS* overall survival, *95 % CI* 95 % confidence interval

No significant prognostic effect of macrophage infiltration was found in our study. Controversial data exist in the literature regarding the role of macrophages: Cai et al. [28] demonstrated association between high density macrophage infiltration and adverse prognostic effect in DLBCL that could be explained by their putative role in the tumor-induced immunosuppression [3]. However, Linderoth et al. described an opposite result with lower macrophage infiltration showing an adverse effect on DLBCL outcome [22]. We could not demonstrate any relevant effect of follicular T cells (CD57+) on survival. Summarizing our observations and the controversial results on nodal DLBCL in respect of the prognostic effect of the different subclasses of T-cells and antigen presenting cells may suggest that functional interactions are rather more important than single ingredients of the immune microenvironment [2,25,27,29].

There is consensus in the literature that translocations involving the MYC oncogene confer an adverse prognosis in patients with nodal DLBCL. The adverse prognostic effect of MYC protein expression independent of MYC translocations has recently been published [6]. MYC protein expression (>30 %) was demonstrated in PB-DLBCL by Koens et al. in 31 % of their cases [16]. In our cohort of PB-DLBCL, MYC positivity >30 and >10 % was observed in 25 and 40 % of the cases, respectively, comparable to the results in nodal DLBCL [6,17]. In our study, no effect of MYC protein expression was observed on OS. Recent data on nodal DLBCL confirmed the negative prognostic effect of BCL2 expression, and in addition, an inferior survival for patients with simultaneous expression of MYC and BCL2 proteins was demonstrated compared to the effects of MYC or BCL2 expression alone [17]. High BCL2 protein expression was found in more than 50 % of the PB-DLBCL cases published, indicating a possible role in the pathogenesis; however, no correlation with survival was found that may be due to the low case number [30]. In contrast, our data on PB-DLBCL is demonstrating significant adverse prognostic effect of BCL2 protein expression independent of MYC protein expression and other risk factors like cell of origin, age, and CD8+ T cell density. BCL2 remained significantly associated with an adverse survival in ABC and GCB derived subgroups of PB-DLBCL.

Several lines of evidence suggest that tumor cell phenotype determined by tumor cell origin provides an important prognostic biomarker for nodal DLBCL; patients with GCB-derived tumor have a longer survival than those with an ABC phenotype [2,4]. Our data on PB-DLBCL shows no significant difference in OS of patients with GCB or ABC-derived tumors. Data in literature regarding the prognostic significance of tumor cell phenotype in PB-DLBCL is controversial. Two studies of PB-DLBCL have shown a better survival for patients with GCB compared to non-GCB type tumors similarly to nodal DLBCL [11,13]. However, Heyning et al. [14] and Bhagavathi et al. [15], studying a comparable number of cases, found a similar favorable survival independent of tumor cell phenotype that is consistent with our result. Differences in observations could be due to the low number of PB-DLBCL cases investigated compared to the studies on DLBCL that are usually performed on large cohorts, or it may represent the different biological nature of the two entities.

In our cohort, an adverse prognostic effect of increased tumor cell proliferation was observed as assessed by Ki67 staining. There are only few immunohistochemical studies in PB-DLBCL using Ki67 staining, and no association was observed between Ki67 expression and survival [11,28]. In previous publications, assessment of tumor cell proliferation yielded conflicting prognostic predictions of patients with DLBCL. A recent report found that elevated Ki67 expression may be associated with a higher relapse rate after CR and inferior event-free survival, but no significant effect was observed on OS [31].

In our cohort of PB-DLBCL patients, disease outcome failed to show any association with the activity of the PI3K/AKT/mTOR pathway. However, a significant correlation was found between ABC phenotype and mTOR activity. This finding is in line with the observation that tumor cell phenotype was also independent of survival of PB-DLBCL patients. mTOR signaling pathway was found to be activated in DLBCL [32], and mTOR inhibitors have been used in cases of relapsed/refractory DLBCL [33]. The elevated activity of the PI3K/AKT/mTOR signal transduction pathway could be an important factor behind the adverse survival of DLBCL patients with activated-B-cell phenotype based on the recent results of Sebestyen et al. [5], showing a significant association between mTOR activity and non-germinal center signature and poor outcome. Our results indicate that the mTOR signaling pathway, in contrast to nodal DLBCL, may have no significant role in PB-DLBCL progression and could also partially explain the more favorable outcome of PB-DLBCL compared to nodal DLBCL. However, the activity of the mTOR signaling pathway may provide an additional therapeutic target for resistant PB-DLBCL with activated B-cell phenotype.

PB-DLBCL is a rare disease with distinct clinicopathological features and a more favorable prognosis compared to nodal DLBCL. Clinical factors like IPI, age, and disease stage at diagnosis show prognostic relevance with respect to disease outcome for DLBCL with nodal or extranodal presentation. However, recent studies on nodal DLBCL emphasize the importance of the host response represented by the T-cell infiltration. This is in accordance with our current results, i.e., that the tumor-infiltrating cytotoxic (CD8+) T-cells may represent a novel factor influencing the survival in PB-DLBCL. Our results also indicate that in contrast to nodal DLBCL, BCL2 protein expression but not MYC is a potential marker for inferior OS in ABC and GCB derived PB-DLBCL.

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Conflict of interest The authors declare that they have no conflict of interest.

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