Comparison of two different immunohistochemical algorithms identifying prognostic subgroups of DLBCL

Joanna Jabłońska[1], Dorota Jesionek-Kupnicka[1], Piotr Potemski[2], Artur Kowalik[3], Jacek Sygut[3], Radzisław Kordek[1]

[1]Department of Pathology, Chair of Oncology, Medical University of Łódź
[2]Department of Chemotherapy, Chair of Oncology, Medical University of Łódź
[3]Department of Pathology, Center of Oncology of Kielce

In this study we analyzed the prognostic value of single and combined immunohistochemical markers, according to algorithms proposed by Hans *et al.* and Muris *et al.* in 66 *de novo* diffuse large B-cell lymphoma (DLBCL) cases. The main aim of our study was to compare usefulness of these two immunohistochemical algorithms for the subdivision of DLBCL into prognostically relevant subgroups. Cases classified as germinal centre B-cell (GCB) had a significantly lower risk of death ($p = 0.008$) compared with the non-GCB group. The 5-year overall survival (OS) rate was 85% for the GCB group and only 30% for the non-GCB group ($p = 0.003$). Furthermore, division into the GCB and non-GCB group predicted prognosis in cases with low International Prognostic Index (IPI) ($p = 0.03$). GCB patients with a low IPI score had a significantly better OS than those from the non-GCB group (93% versus 45%) ($p = 0.02$). Although the 5-year OS of favourable group 1 from Muris algorithm was slightly better than in group 2, the difference was not significant ($p = 0.241$). In summary, our results indicate that the algorithm of Hans *et al.* has a significantly better prognostic value. By using immunohistochemistry and this algorithm, we can subclassify DLBCL into prognostically distinct subgroups and further refine the prognosis based on IPI.

Key words: diffuse large B-cell lymphoma, immunohistochemical algorithms, prognosis.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is one of the most common subtypes of non-Hodgkin's lymphomas of adults and accounts for approximately 40% of cases [1]. It is a clinically, morphologically and genetically heterogeneous group of tumours [1]. This heterogeneity is well reflected by the clinical course of the disease. 40% of patients with diffuse large B-cell lymphoma respond well to the current therapy and show long-term survival, but at least 50% have relapse after conventional therapy [2]. The most effective clinical tool for predicting the outcome of patients with DLBCL is still the International Prognostic Index (IPI), which identifies subgroups of patients with very poor or good prognosis [3]. Although the importance of IPI was validated in many studies [3, 4], it is less helpful for predicting treatment response in individual patients. The index alone is insufficient to distinguish between patients who should be cured with conventional therapy and those who will have relapse or progressive disease and require more aggressive therapy.

Because the heterogeneity of DLBCL has an impact on the clinical course of the disease and patients' response to conventional methods of treatment, it was necessary to find a useful tool, independent of the IPI, for predicting outcome and optimizing the treatment. Therefore, with the use of different techniques, investigators tried to subclassify DLBCLs and to identify prognostically important groups of patients.

Alizadeh *et al.* showed diversity in gene expression among DLBCLs and identified molecularly distinct forms of DLBCL, which had gene expression patterns indicative of different stages of B-cell differentiation [5]. According to cDNA microarray-based gene expression profiling, DLBCL was divided into 3 pro-

gnostically important subgroups [5]. Patients from the germinal centre B-cell (GCB)-like subgroup of DLBCL had significantly better overall survival compared to those from the activated B-cell (ABC)-like subgroup [5]. This prognostic significance was independent of IPI [5-7]. The third type of the gene expression profile (a group of unclassified cases), described by Alizadeh *et al*., was a heterogeneous and poorly described subtype [5]. This type was associated with a poor outcome similar to the ABC group [5, 6], and was later classified as the non-germinal centre group [6].

The results of their study was further confirmed by others [6-8]. However, the cDNA microarray technique is expensive, time-consuming and generally unavailable routinely because it depends on the availability of fresh frozen samples. Therefore, this method is impractical as a clinical tool. In an attempt to find a simpler and more practical method of DLBCL subclassification, the investigators started using immunohistochemistry in their studies [9-15]. As a result of these studies, two immunohistochemical algorithms have been proposed [9, 10]. The first one, proposed by Hans *et al*. [9] (Fig. 1A) was based on expression of CD10, Bcl-6 and MUM1. With the use of these combined markers the authors divided DLBCLs into GCB and non-GCB subgroups. They noticed markedly better survival of patients in the GCB group than those in the non-GCB group [9]. The second algorithm [10] (Fig. 1B) identifying two prognostically important subtypes of DLBCL was later proposed by Muris *et al.* and was also based on expression of three markers: Bcl-2, CD10 and MUM1 [10]. Authors of this study [10] suggested that this algorithm had a stronger prognostic value than the previous one.

In our study, we have investigated immunohistochemically pretreatment, diagnostic samples from 66 DLBCL cases in attempt to compare these 2 algorithms and establish which markers and algorithm are most useful for the subdivision of DLBCL into prognostic relevant subgroups.

Material and methods

Patients

Formalin-fixed, paraffin-embedded tissue block specimens of 66 DLBCLs, sampled between 2000 and 2006, were selected from the archives of the Department of Pathology, Chair of Oncology of the M. Kopernik Memorial Hospital in Łódź, Poland. Only specimens obtained at the time of diagnosis, before initiation of any treatment were included. All cases were reviewed by morphologic features and immunohistochemical staining to verify that all specimens were DLBCL according to the present WHO classification system. Patients with the diagnosis of follicular lymphoma or other type of indolent lymphoma with transformation into diffuse large B-cell lymphoma were not included. Cases were selected only on the basis on availability of complete clinical information and sufficient histological material. The clinical records were reviewed in all of the DLBCL patients with particular reference to age at diagnosis, site of initial involvement, Ann Arbor stage at presentation, response to treatment, achievement of complete remission (CR), the occurrence of relapse or progression and survival. The follow-up period was counted from the day of diagnosis until death or the date of the last follow-up for living (censored) patients.

TMA blocks construction

For the tissue microarray (TMA), haematoxylin and eosin-stained sections from each paraffin-embedded, formalin-fixed block were used to define diagnostic areas, and 3 random, representative 1 mm cores were obtained from each case, using a puncher tissue microarrayer (1 mm punch set, code: MP10; Beecher Instruments, Silver Spring, MD). The construction (protocol) of the TMA block was compiled from two articles [16, 17]. Briefly each retrieved tissue core was trimmed minimally at one end with a razor blade and then was attached on the adhesive platform (prepared from double-sided adhesive tape attached to computer-generated paper grid and x-ray film). The array of tissue cores was embedded in an embedding mould, which was filled with liquid paraffin. Then the blocks were cooled, the adhesive platform was peeled off, and after this, the cutting surface was ready for sectioning (TMA blocks were prepared in the Department of Pathology, Centre of Oncology of Kielce).

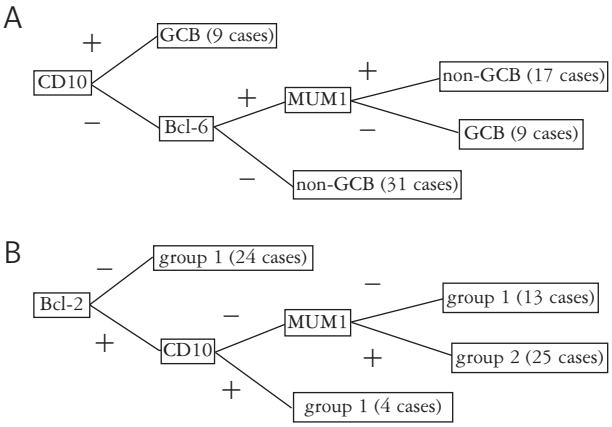

Fig. 1. Immunohistochemical algorithms identifying prognostically important subgroups of DLBCL: A – proposed by Hans *et al;* B – proposed by Muris *et al.*

Table I. Antibodies used for immunohistochemical stains with pretreatments and dilutions

ANTIBODY	CLONE	SOURCE	DILUTION	ANTIGEN RETRIEVAL
CD20	L26	Dako, Glostrup, Denmark	1 : 400	EDTA 20
CD10	56C6	Novocastra, Newcastle upon Tyne, UK	1 : 50	EDTA 20
Bcl-6	PG-B6p	Dako, Glostrup, Denmark	1 : 20	EDTA 40
Bcl-2	124	Dako, Glostrup, Denmark	1 : 50	EDTA 20
MUM1	MUM1p	Dako, Glostrup, Denmark	1 : 50	EDTA 20
Ki67	MIB-1	Dako, Glostrup, Denmark	1 : 100	EDTA 20
p53	DO-7	Dako, Glostrup, Denmark	–	EDTA 20

EDTA 20 indicates 20 minutes at 97°C in Tris/EDTA buffer, pH 9 (Dako Target Retrieval Solution, pH 9), EDTA 40 indicates 40 minutes at 97°C in Tris/EDTA buffer, pH 9.9 (Dako Target Retrieval Solution, pH 9.9)

Immunohistochemistry

Sections (2 micrometers) were cut from each TMA block and stained with antibodies.

The following markers were used: CD20, CD10, Bcl-6, Bcl-2, MUM1/IRF4, Ki67, p53. Formalin-fixed paraffin sections were previously deparaffinized and rehydrated. Heat-induced antigen retrieval was performed. Endogenous peroxidase activity was blocked. Slides were incubated for 20 to 40 minutes with primary antibodies and then the DAKO Envision+ System HRP (DAB) was used as a secondary antibody and as a chromogen. Sections were counterstained with Mayer haematoxylin. The immunoreaction was done in an automated Dako Autostainer Plus (Dako, Glostrup, Denmark). The source of the antibodies, dilution used and antigen retrieval procedures employed are shown in Table I.

Cases were considered as positive for CD10, Bcl-6, MUM1 and p53 if 30% or more of the tumour cells were stained with antibody, whereas for Bcl-2, a cut-off level of 50% positive cells was used. The cut-off values for positive staining were established according to studies of the recent literature [9-12, 21]. We used results from the CD10, Bcl-6, Bcl-2 and MUM1 staining to divide all DLBCL cases into subgroups according to two different algorithms proposed by Hans *et al.* (Fig. 1A) and Muris *et al.* (Fig. 1B).

Statistical analysis

Overall survival was calculated according to the Kaplan-Meier method from the date of primary diagnosis to the date of death or the last follow-up whereas disease-free survival was calculated to the date of recurrence or the last follow-up. Differences in survival distributions were evaluated by a log-rank test. Univariate and multivariate survival analyses were performed with the use of the Cox's proportional hazard regression model. Pearson's χ^2 test or Fisher exact test were used to compare proportional data between groups. All results were considered statistically significant when two-sided p was less than 0.05. The analyses were performed using the StatsDirect software (StatsDirect Ltd., the United Kingdom).

Results

Patient characteristics

Clinical data were available for all 66 patients and are summarized in Table III. As the International Prognostic Index (IPI) was evaluated retrospectively not, all factors were available for all patients. Complete information of the IPI was available in 58 of the 66 cases. All patients were treated with CHOP-based chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone), none of them received anti-CD20 antibody (rituximab) because all cases in this series were treated before introduction of rituximab as a routine treatment of DLBCL in Poland. The median follow-up period for all patients was 37 months (range 1-97 months), for censored patients it was 43.5 months (range 20-97 months). 34 patients are still alive, 31 have died of the disease and 1 of unrelated cause. The 5-year OS rate for the entire group was 44.9%. Median survival time was 45 months.

The results of the univariate analysis of overall survival are shown in Table II. Younger age, low Ann Arbor stage at presentation, low IPI score (0-2), achieving of CR were all associated with a decreased relative risk of death (Table II). The primary site of the disease (nodal vs. extranodal), the gender of patients, B symptoms, level of LDH and B2-microglobulin did not show a significant impact on OS (Table II).

Expression of the single and combined immunohistochemical markers

Expression of CD10 was seen in 14% (9/66) of cases, Bcl-6 in 53% (35/66), MUM1 in 55% (36/66), Bcl-2 in 64% (42/66) and p53 in 26% (17/66). Rep-

Table II. Results of univariate survival analysis – prognostic value of immunohistochemical stains, immunohistochemical algorithms and clinical features

PARAMETER	NO. OF CASES	HR	95% CI	P
CD10 (positive vs. negative)	9 vs. 57	0.163	0.022-1.194	0.074
Bcl-6 (positive vs. negative)	35 vs. 31	0.523	0.257-1.063	0.073
Bcl-2 (positive vs. negative)	42 vs. 24	1.309	0.618-2.773	0.482
MUM1 (positive vs. negative)	36 vs. 30	1.113	0.553-2.241	0.763
p53 (positive vs. negative)	17 vs. 49	1.604	0.756-3.404	0.218
non-GCB group vs. GCB group	18 vs. 48	5.065	1.537-16.691	0.007
group 2 vs. group 1 (Muris algorithm)	41 vs. 25	1.515	0.748-3.067	0.248
IPI high vs. low	30 vs. 38	2.305	1.413-3.761	0.001
ann Arbor stage (IV vs. III vs. II vs. I)	15/15/19/17	1.532	1.114-2.107	0.008
ann Arbor stage (III/IV vs. I/II)	30 vs. 36	1.843	0.908-3.738	0.091
age ≥60 years vs. <60 years)	41 vs. 25	2.782	1.244-6.222	0.013
remission (not-CR vs. CR)	23 vs. 43	13.242	5.581-31.418	< 0.001

HR – hazard ratio of death, 95% CI – 95% confidence interval for HR

resentative examples of immunohistochemical staining are shown in Figure 2. The univariate analysis showed a distinct trend, although not significant, between expression of CD10 and longer OS (p = 0.074) Similar relationship was observed between expression of Bcl-6 and longer OS (p = 0.073). Expression of other markers: MUM1, Bcl-2 and p53 did not predict OS.

Using the algorithm of Hans *et al.* based on the expression of CD10, Bcl-6 and MUM1 we divided 66 cases into two groups (Fig. 1A). Non-GCB group was more common phenotype – 73% (48 of 66 cases) than GCB group – 27% (18 of 66 cases). In the GCB group, 50% (9/18) expressed both markers (CD10 and bcl-6), whereas 50% (9/18) expressed Bcl-6 alone. In our study, there was no case which had CD10 expression alone. MUM1 was expressed in 22% (4/18) cases of GCB group. Of the non-GCB cases, 31% (15/48) expressed MUM1 alone, 35% (17/48) expressed both MUM1 and Bcl-6 and 33% (16/48) were negative for both markers. Bcl-2 positive staining was seen in 42 cases, 11 of them (26%) belong to GCB group and 31 to non-GCB group (74%). Expression of bcl-2 was not associated with a significant difference in OS in both groups (data not shown).

Using the alternative algorithm proposed by Muris *et al.* based predominantly on expression of Bcl-2, CD10 and Bcl-6 we divided 66 cases to favourable group 1 and to unfavourable group 2 (Fig. 1B). Of the 66 cases, 41 (62%) were classified as favourable group 1 and 25 cases (38%) as unfavourable group 2.

The clinical features of the patients classified with TMA as GCB/non-GCB group and group 1/2 phenotype are summarized in Table III.

Prognostic value of two immunohistochemical algorithms

Cases classified by TMA as GCB had a significantly better OS (p = 0.008) compared with non-GCB group (Fig. 3). The 5-year OS rate for the GCB was 83.0% compared with only 30.2% for the non-GCB group (p = 0.003). For the entire group, patients that presented low IPI scores had significantly better OS (p = 0.001) than those who presented high IPI scores. When considering those cases (patients with low IPI and with high IPI) separately while applying to them the GCB/non-GCB division, the 5-year OS for patients in GCB group with low IPI was 92.9% vs. 45.4% for patients in non-GCB group and low IPI (p = 0.021) (Fig. 3). Similar analysis among patients with high IPI scores was impossible to perform because the number of patients in GCB group was too small. After comparing Muris *et al.* division with Hans *et al.* classification, 23 non-GCB cases were classified as favourable group 1 (according to Muris algorithm). This could be the explanation of the slightly worse survival of patients with group 1 category (5-year OS rate 49.9%) than patients in GCB group (5-year OS 83.0%) (Fig. 3). However, in all patients there was no statistically significant difference in OS between group 1 and group 2 category.

Multivariate analysis

Cox regression multivariate analysis of OS was performed in 60 cases with complete information on the IPI risk group, Ann Arbor stage, status of remission, Hans GCB/non-GCB groups and group 1/2 phenotype (according to Muris *et al*.). In the final model, only IPI retained independent prognostic significance

Table III. Clinical features depending on the results of the algorithm of Hans (GCB/non-GCB) and the algorithm of Muris (group 1/group 2)

	No. of cases (%)	GCB (%)	Non-GCB (%)	P	Group 1 (%)	Group 2 (%)	p
Total no.	66 (100)	18 (27)	48 (73)	–	41 (62)	25 (38)	–
Sex							
male	30 (45)	8 (44)	22 (46)	0.999	18 (44)	12 (48)	0.746
female	36 (55)	10 (56)	26 (54)		23 (56)	13 (52)	
Age, years							
median	64	59	66	–	63	65	–
range	22-100	22-100	35-88		22-100	35-88	
Age							
<60 years	25 (38)	9 (50)	16 (33)	0.214	16 (39)	9 (36)	0.806
≥60 years	41 (62)	9 (50)	32 (67)		25 (61)	16 (64)	
Ann Arbor stage							
I/II	36 (55)	16 (89)	20	0.001	23 (56)	14 (56)	0.994
III/IV	30 (45)	2 (11)	28		18 (44)	11 (44)	
Ann Arbor stage							
I	17 (26)	9 (50)	8 (17)	0.002	13 (32)	4 (16)	0.427
II	19 (29)	7 (39)	12 (25)		10 (24)	9 (36)	
III	15 (23)	2 (11)	13 (27)		8 (20)	7 (28)	
IV	15 (23)	0 (0)	15 (31)		10 (24)	5 (20)	
B symptoms							
yes	18 (27)	2 (11)	16 (33)	0.119	10 (24)	8 (32)	0.501
no	48 (73)	16 (89)	32 (67)		31 (76)	17 (68)	
Localization							
nodal	49 (74)	11 (61)	38 (79)	0.205	28 (68)	21 (84)	0.157
extranodal	17 (26)	7 (39)	10 (21)		13 (32)	4 (16)	
LDH							
normal	13 (20)	6 (33)	7 (15)	0.156	8 (20)	5 (20)	0.999
high	45 (68)	10 (56)	35 (73)		28 (68)	17 (68)	
IPI risk							
low (0-2)	30 (45)	13 (72)	17 (35)	0.008	18 (44)	12 (48)	0.737
high (3-5)	28 (42)	3 (17)	25 (52)		18 (44)	10 (40)	
Complete remission							
yes	43 (65)	17 (94)	26 (54)	0.003	28 (68)	15 (60)	0.493
no	23 (35)	1 (6)	22 (46)		13 (32)	10 (40)	

($p = 0.023$) but there was a clear tendency towards significance of Hans algorithm ($p = 0.072$) with a hazard ratio of death 4.00 (95% CI: 0.88-18.11) for non-GCB group vs. GCB group. None of other variables predicted an clinical outcome independently.

Discussion

Diffuse large B-cell lymphoma is one of the most common non-Hodgkin's lymphomas but it is also one of the most heterogeneous [1]. Because this heterogeneity has an impact on the clinical course of the disease and patient response to treatment [2] it was necessary to find a useful tool for predicting outcome and making a treatment decision beyond the IPI.

In this study we analyzed prognostic value of the single and combined immunohistochemical markers of 66 *de novo* DLBCL cases. The main aim of our study was to evaluate usefulness of two different immunohistochemical algorithms [9, 10] for predicting outcome.

Our results indicate that the algorithm proposed by Hans *et al*. [9] has better prognostic value for DLBCL cases than the alternative algorithm [10]. Cases classified as GCB had a significantly better OS compared with non-GCB group. The 5-year OS for the GCB group was 83% compared with only 30% for the non-GCB group. The division into GCB and non-GCB group also predicted prognosis independently from IPI. Patients with low IPI scores from GCB group had a significantly better OS than those from non-GCB group (93% vs. 45%). Although the 5-year OS of favourable group 1 from Muris algorithm was slightly better than that of group 2 (50% vs. 37%), this difference did not achieve statistical significance.

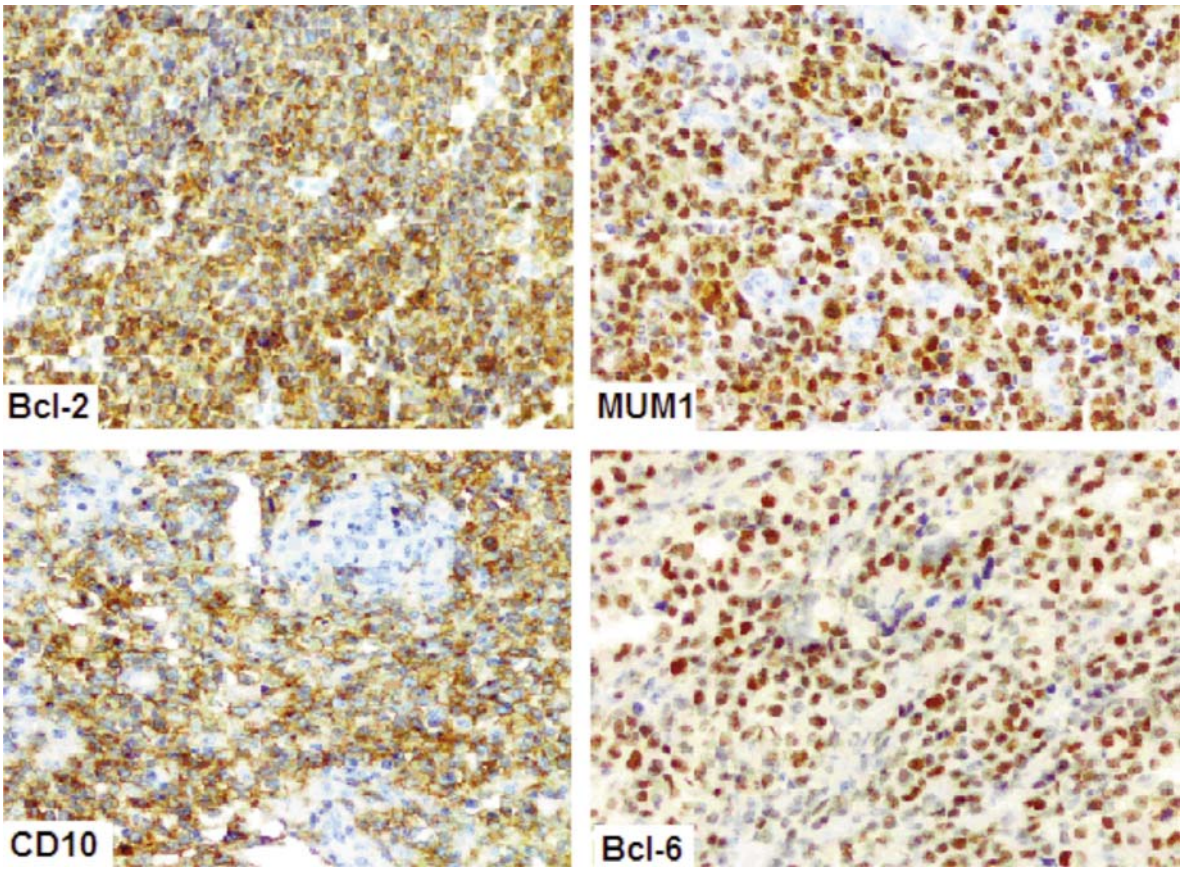

Fig. 2. Examples of the immunohistochemical positive staining of markers:
Bcl-2 – expression showing membranous staining pattern (original magnification 200×), MUM1 – expression showing nuclear/cytoplasmic staining pattern (original magnification 200×), CD10 – expression showing membranous staining pattern (original magnification 200×), Bcl-6 – expression showing nuclear staining pattern (original magnification 200×)

Hans' algorithm [9] is based on the expression of 3 markers: CD10, Bcl-6 and MUM1. CD10 is a proteolytic enzyme which expression is restricted to germinal centre cells [18]. Several studies have examined the prognostic significance of CD10 positivity in DLBCL with contradicting results. Some of these studies reported an association between CD10 expression and a better prognosis [9, 11, 12, 15, 19-22] contrary to other studies that had not found any difference in outcome between CD10+ and CD10- tumours [13, 23]. These discrepant findings could depend on different techniques used in the identification of an antigen and different numbers of examined patients. In the studies using immunohistochemistry on paraffin sections and the largest groups of patients [12, 19, 21], CD10 expression was correlated with improved overall survival and predicted prognosis in the group of low IPI [19]. In our study there was a strong tendency between expression of CD10 and better OS. However, these conflicting data suggest that using CD10 alone may not be useful in the prediction of survival in DLBCL.

The second GCB marker, Bcl-6 acts as a transcriptional repressor and represses genes involved in lymphocyte activation and differentiation, cell cycle control and inflammation [24]. Bcl-6 expression in DLBCL tends to be the single most important predictive factor of good prognosis. Most studies have suggested that expression of Bcl-6 is associated with better prognosis and it might be a useful prognostic marker in DLBCL [9, 11, 21, 22, 26], however, in some studies the difference was not at a statistically significant level [15, 20]. Only in Colomo *et al*. study [13], investigators had not found any difference in overall survival depending on Bcl-6 expression. These differences could be explained by the differences in the cut off value, in staining techniques and may be related to the heterogeneity of the examined group of patients. Because the variable number of tumour nuclei are positive, investigators used a different cut-off value (from 10% to 30%) for positive staining. Previously, 10% have been most commonly chosen [11, 12, 20] but further studies have shown that this level might be too low to subdivide DLBCLs into appropriate and reproducible subgroups of patients [9] and now in most studies [21, 22, 25] 30% is considered as a better cut-off value. These findings suggest that using this antigen alone as a prognostic marker may give divergent results. The last marker from Hans algorithm [9],

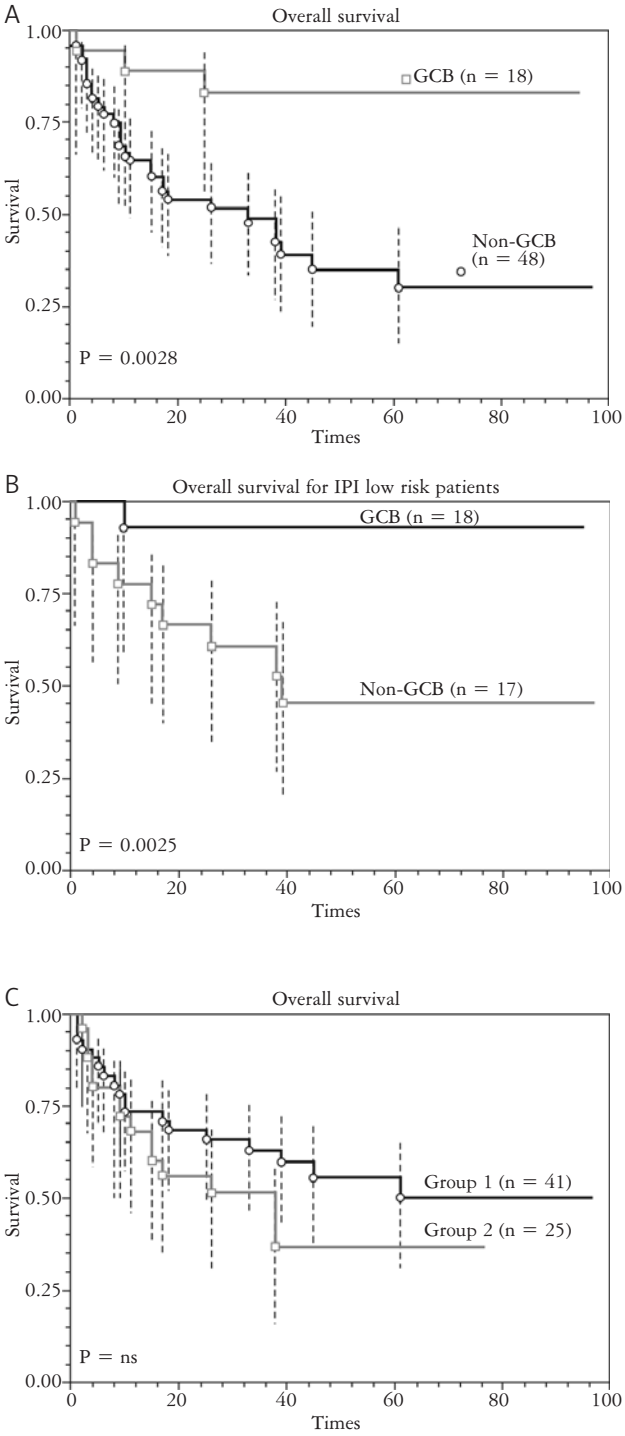

Fig. 3. Overall survival time for:
A – the GCB subgroup (upper line) compared with the non-GCB subgroup (lower line) based on the algorithm of Hans *et al.*
B – patients with low IPI scores and the GCB subgroup (upper line) compared with the non-GCB subgroup (lower line)
C – 'favourable' group 1 (upper line) compared with 'unfavourable' group 2 (lower line) based on the algorithm of Muris *et al.*

MUM1/IRF4 is expressed in plasma cells and in small subset of germinal centre cells [27]. The studies that evaluated prognostic significance of MUM1 expression in DLBCL brought different results. Hans *et al.* [9] have reported that expression of MUM1 in at least 30% of tumour cells is associated with significantly worse outcome [9], which was supported by Chang *et al.* [15]. But other studies did not found any correlation between MUM1 expression and overall survival [11, 13, 20, 21] or this relation did not achieve a statistical level [22]. These differences could be partially explained by the use of monoclonal antibody by Hans *et al.*, whereas others used a polyclonal one.

These different findings suggest that any of these 3 markers used alone does not give a sufficiently certain prognostic value but after connecting them together (according to the algorithm proposed by Hans *et al.*) a useful tool can be constructed to identify prognostically important subgroups of DLBCL.

Although many studies have reported that this three-marker model is useful to identify non-GCB or GCB phenotype which is thought to have a strong prognostic significance for patients with DLBCL, there are studies that did not confirm this observation [13]. Therefore, during last few years there have been attempts to improve Hans' algorithm and to propose an alternative one. Results of these efforts were as follows: a new algorithm proposed by Muris *et al*. [10], division into 3 groups in Chang *et al*. study [15] and an attempt to improve Hans' algorithm made by Amen *et al*. [22]. The best known and most often researched is Muris *et al*. algorithm [10]. It is based on expression of the antiapoptotic protein, Bcl-2 added to CD10 and MUM1 markers. Muris *et al.* [10] has divided DLBCL into favourable group 1 and unfavourable group 2 and has suggested that this algorithm has a stronger prognostic value than the previous one [9]. In our study we decided to check it.

Evaluation of Bcl-2 expression was a basic difference between these two algorithms. The prognostic value of this marker expression was the subject of numerous studies. Several studies reported that high expression of Bcl-2 was an adverse prognostic factor [11, 12, 22, 28, 29] alone and also in connection with other factors, but other studies had not found any statistically significant difference in overall survival [30, 31]. An influence of Bcl-2 on OS independent of IPI was reported only by Barrans *et al.* [12] and Gascoyne *et al*. [29]. These different results could arise due to absence of the determined cut-off value. The investigators did not use the same criteria to classify Bcl-2 positive or negative cases and the fluctuation of cut-off point was within the 10% to > 50% range. However, most studies indicate that a higher cut-off value is related with more significance of marker expression [32]. In more recent studies authors have suggested that expression of Bcl-2 is an adverse prognostic factor only in non-GCB subgroup [21, 28]. In our study the expression of Bcl-2 did not relate with OS equally in the entire group of patients and in non-GCB subgroup.

The subclassification of DLBCL into the GCB type and ABC type using gene expression profiling or immunohistochemical staining was made before the era of immunochemotherapy. It was reported that the GCB type of DLBCL showed a significantly better overall survival than the ABC type; however, the patients were treated with the CHOP regimen without rituximab. Recently published studies have indicated improvement in clinical outcome in non-germinal centre type of DLBCL and elimination the differences between GCB and non-GCB DLBCL after addition of rituximab to the CHOP regimen [33-35]. With the well-accepted addition of Rituximab to the typical large B-cell lymphoma chemotherapeutic regimen, revalidation of any survival differences between large B-cell lymphoma subtypes is necessary and prospective clinical trials are needed.

Because of diversity of the results received in different studies and introduction of rituximab to standard regimens, dividing DLBCL into prognostically important subgroups independently of IPI seems to be a complex and multifaceted problem. The aim of our study was to compare the two best known prognostic algorithms and the results indicate unequivocally the benefit of the algorithm originally describedby Hans *et al*. It has a significant impact on DLBCL prognosis and this division is independent of IPI score.

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References

1. Gatter KC, Warnke RA. Diffuse large B-cell lymphoma. In: Jaffe ES, Harris NL, Stein H, Vardiman JW (ed). World Health Organization Classification of Tumours, Pathology and Genetics. IARC Press, Lyon 2001; 171-174.
2. Hennessy BT, Hanrahan EO, Daly PA (2004) Non–Hodgkin Lymphoma: an update. Lancet Oncol, 2004; 5: 341-353.
3. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project N Engl J Med., 1993; 329: 987-994.
4. Blay J, Gomez F, Sebban C, Bachelot T, Guglielmi C, Hagenbeek A, Somers R, Chauvin F, Philip T (1998) The International Prognostic Index correlates to survival in patients with aggressive lymphoma in relapse: analysis of the PARMA trial. Parma Group. Blood 1993; 92(10): 3562-3568.
5. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature, 2000; 403: 503-511.
6. Rosenwald A, Wright G, Chan WC, et al. Lymphoma/ Leukemia Molecular Profiling Project. The use of molecular profiling to predict survival after chemotherapy for diffuse large B-cell lymphoma. N Engl J Med 2002; 346: 1937-1947.
7. Wright G, Tan B, Rosenwald A, et al. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. Proc Natl Acad Sci U S A 2003; 100: 9991-9996.
8. Shipp MA, Ross KN, Tamayo P, et al. Diffuse large B-cell lymphoma outcome prediction by gene expression profiling and supervised machine learning. Nat Med 2002; 8: 68-74.
9. Hans CP, Weisenburger DD, Vose JM, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 2004; 103: 275-282.
10. Muris JJ, Meijer CJ, Vos W, et al. Immunohistochemical profiling based on Bcl-2, CD10, and MUM1 expression improves risk stratification in patients with primary nodal diffuse large B cell lymphoma. J Pathol 2006; 208: 714-723.
11. Berglund M, Thunberg U, Amini RM, et al. Evaluation of immunophenotype in diffuse large B-cell lymphoma and its impact on prognosis. Modern Pathology 2005; 18: 1113-1120.
12. Barrans SL, Carter I, Owen RG, et al. Germinal center phenotype and bcl-2 expression combined with the International Prognostic Index improves patient risk stratification in diffuse large B-cell lymphoma. Blood 2002; 99: 1136-1143.
13. Colomo L, López-Guillermo A, Perales M, et al. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. Blood 2003; 101: 78-84.
14. Biasoli I, Morais JC, Scheliga A, et al. CD10 and Bcl-2 expression combined with the International Prognostic Index can identify subgroups of patients with diffuse large B-cell lymphoma with very good or very poor prognoses. Histopathology 2005; 46: 328-333
15. Chang CC, McClintock S, Cleveland RP, et al. Immunohistochemical expression patterns of germinal center and activation B-cell markers correlate with prognosis in diffuse large B-cell lymphoma. Am J Surg Pathol 2004; 28: 464-470.
16. Chen N, Zhou Q. Constructing tissue microarrays without prefabricating recipient blocks. Am J Clin Pathol 2005; 124: 103-107.
17. Pires ARC, Andreiuolo FM, de Souza SR. TMA for all: a new method for the construction of tissue microarrays without recipient paraffin block using custom-built needles. Diagnostic Pathology 2006; 1-14.
18. McIntosh GG, Lodge AJ, Watson P, et al. NCL-CD10-270: a new monoclonal antibody recognizing CD10 in paraffin-embedded tissue. Am J Pathol 1999; 154: 77-82.
19. Ohshima K, Kawasaki C, Muta H, et al. CD10 and Bcl10 expression in diffuse large B-cell lymphoma: CD10 is a marker of improved prognosis. Histopathology 2001; 39: 156-162.
20. Young-Ha O, Chan-Kum P. Prognostic evaluation of nodal diffuse large B cell lymphoma by immunohistochemical profiles with emphasis on CD138 expression as a poor prognostic factor. J Korean Med Sci 2006; 21: 397-405.
21. Sjö LD, Poulsen CB, Hansen M, et al. (2007) Profiling of diffuse large B-cell lymphoma by immunohistochemistry: identification of prognostic subgroups. Eur J Haemathol 2007; 79: 501-507.
22. Amen F, Horncastle D, Elderfield K, et al. Absence of cyclin-D2 and Bcl-2 expression within the germinal centre type of diffuse large B-cell lymphoma identifies a very good prognostic subgroup of patients. Histopathology 2007; 51: 70-79.
23. Fabiani B, Delmer A, Lepage E, et al.; Groupe d'Etudes des Lymphomes de l'Adulte (2004) CD10 expression in diffuse large B-cell lymphomas does not influence survival. Virchows Arch 2004; 445: 545-551.
24. Shaffer A, Yu X, He Y, et al. Bcl-6 represses genes that function in lymphocyte differentiation, inflammation and cell cycle control. Immunity 2000; 13: 199-212.
25. Zinzani PL, Dirnhofer S, Sabattini E, et al. Identification of outcome predictors in diffuse large B-cell lymphoma. Immunohistochemical profiling of homogeneously treated de

novo tumors with nodal presentation on tissue micro-arrays. Haematologica 2005; 90: 341-347.
26. Lossos IS, Jones CD, Warnke R, et al. Expression of a single gene, Bcl-6, strongly predicts survival in patients with diffuse large B-cell lymphoma. Blood 2001; 98: 945-951.
27. Falini B, Fizzotti M, Pucciarini A, et al. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells and activated T cells. Blood 2000; 95: 2084-2092.
28. Iqbal J, Neppalli VT, Wright G, et al. Bcl-2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. J Clin Oncol 2006; 24: 961-968.
29. Gascoyne RD, Adomat SA, Krajewski S,et al. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. Blood 1997; 90: 244-251.
30. Zhang A, Ohshima K, Sato K, et al. Prognostic clinicopathologic factors, including immunologic expression in diffuse large B-cell lymphomas.Pathol Int 1999; 49: 1043-1052.
31. Hill ME, MacLennan KA, Cunningham DC, et al. Prognostic significance of Bcl-2 expression and Bcl-2 major breakpoint region rearrangement in diffuse large cell non-Hodgkin's lymphoma: a British National Lymphoma Investigation Study. Blood 1996; 88: 1046-1051.
32. De Leval, Harris NL. Variability in immunophenotype in diffuse large B-cell lymphoma and its clinical relevance. Histopathology 2003; 43: 509-528.
33. Farinha P, Sehn L, Skinnider B, et al. Addition of rituximab to CHOP improves survival in the non-GCB subtype of diffuse large B-cell lymphoma. Blood 2006; 108: 816.
34. Hunt KE, Reichard KK (2008) Diffuse large B-cell lymphoma. Arch Pathol Lab Med 2008; 132: 118-124.
35. Nyman H, Adde M, Karjalainen-Lindsberg ML, et al. Prognostic impact of immunohistochemically defined germinal center phenotype in diffuse large B-cell lymphoma patients treated with immunochemotherapy. Blood 2007; 109: 4930-4935.

Address for correspondence

Joanna Jabłońska
Department of Pathology
Chair of Oncology, Medical University of Łódź
ul. Paderewskiego 4
93-509 Łódź
phone +48 42 689 57 81
fax +48 42 689 57 82
e-mail: joannajab@o2.pl