Poor outcome in patients with diffuse large B-cell lymphoma is associated with high percentage of bcl-2 and Ki 67-positive tumor cells

Visok procenat bcl-2 i Ki-67 pozitivnih tumorskih celija udružen je sa lošijom prognozom kod bolesnika sa difuznim krunopočelijskim B- limfomom

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Abstract

Background/Aim. Newly diagnosed patients with diffuse large B-cell lymphoma (DLBCL) treated with immunochemotherapy have durable remission and improved overall survival. It is important to identify high risk patients who may benefit from even more effective therapies. Methods. In a group of 50 newly diagnosed patients with DLBCL, treated with CHOP/R-CHOP (cyclophosphamide doxorubicine, vincristine, prednisone with or without rituximab) regimen, we analyzed the prognostic value of the expression of Ki67 and bcl-2 at diagnosis as well as other standard clinical parameters: International Prognostic Index (IPI), bulky disease, extranodal distribution and lactat dehydrogenase (LDH). Significance was tested according to response rate and overall survival. Results. Univariate survival analysis showed that high IPI had a statistically significant negative influence on overall and event free survival time (log rank, p < 0.01). The log rank test analysis signified that patients with a high proliferative fraction (Ki-67 ≥ 60%) had a worse overall survival rate (OS5y) of 40% compared to those with low proliferation (Ki-67 < 60%) with OS5y of 80% (p < 0.01). There was a clear difference between bcl-2 positivity (threshold 50%) and the achievement of complete remission (66% vs 86% in patients with bcl-2 high and low levels respectively, p < 0.05). In survival analysis, patients with low bcl-2 expression had significantly higher OS5y - 68% compared to those with high bcl-2+ with OS5y 37% (p < 0.05). Multivariate analysis performed by Cox model revealed that IPI > 3, high Ki-67+ and bcl-2+ positivity had a significant independent prognostic value concerning overall survival (p < 0.05). Conclusion. An initial high IPI score associated with high Ki-67+ and bcl-2+ could represent possible predictive factors of poor prognosis, which would help to identify a high risk subgroup of newly diagnosed DLBCL.

Key words: lymphoma, b-cell; genes, bcl-2; ki-67 antigen; prognosis; antineoplastic combined chemotherapy protocols.

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Evrot /Cijel. Novodijagnostikovani bolesnici sa difuznim krunopočelijskim B-limfomom (DBKL) lečeni imunobemioterapijom imaju duge remisije i bolje ukupno preživljanje. Važno je otkriti bolesnike sa visokim rizikom kojima primena efikasnijeg terapijskog pristupa može pomoći. Metode. U grupi od 50 novodijagnostikovanih bolesnika sa DBKL lečenih protokolom CHOP/R-CHOP (ciklofosfamid, dokorubicin, vincristin, prednizon sa ili bez rituximabla), analizirali smo prostogutikučnost preživljenja Ki-67 i bcl-2 u trenutku postavljanja dijagnoze, uključujući i kliničke parametre: internacionalni prognostični indeks (IPI), voluminoznu tumorsku masu, ekstranodalnu lokalizaciju bolesti, nivo laktat-dehidrogenaze. Njihova značajnost testirana je u odnosu na terapijski odgovor i ukupno preživljanje. Rezultati. Univarijantna analiza preživljenja pokazala je da visok IPI ima statistički značajan negativan uticaj na event-free survival i ukupno preživljanje (logrank test, p < 0.01). Analiza logrank testom pokazala je da bolesnici sa DBKL koji imaju visok proliferativni indeks (Ki-67 > 60%) imaju lošije ukupno preživljanje u odnosu na one sa niskom proliferacijom (Ki-67 < 60%) (40% : 80%, p < 0.01). Postoji jasna razlika između bcl-2 pozitivnosti (granična vrednost 50%) i postizanja potpune remisije (66% : 86% između bcl-2 pozitivnih i negativnih bolesnika, p < 0.05). U analizi preživljanja bcl-2 negativni bolesnici imaju značajno bolje preživljanje u odnosu na bcl-2 pozitivne bolesnike (68% prema 37%, p < 0,05). Multivarijantna analiza izvedena prema Kolkovom modelu pokazala je da IPI > 3, visoka Ki-67 i bcl-2 pozitivnost imaju značajan nezavisni prognoštenički uticaj u pogledu ukupnog preživljanja. (p < 0,05). Zaključak. Visoka vrednost IPI u trenutku postavljanja dijagnoze udružena sa visokim vrednostima Ki-67 i bcl-2 potencijalno mogu predstavljati faktore lošije prognoze koji mogu pomoći u otkrivanju visokorizičnih bolesnika sa DBKL dijagnozom.
Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of Non-Hodgkin lymphoma (NHL) accounting for 30% of all newly diagnosed cases. The CHOP (cyclophosphamide, doxorubicine, vincristine, prednisone) regimen alone or with rituximab (CHOP/R) is standard therapeutic approach for the most patients who have DLBCL. Even with current treatment approaches, a substantial minority of patients (about 30%) are not cured. The International Prognostic Index (IPI) is the most important tool for predicting response to treatment for DLBCL. A substantial variability in outcome has been observed despite IPI subgroups. Thus, identifying new prognostic parameters might contribute towards better prediction of outcome and the development of effective risk-adaptive strategies.

In all clinical models, including the IPI index, there was marked residual heterogeneity in outcome, as reflected by the considerably variable survival of patients with identical prognostic scores. The latter was attributed to the marked genetic and molecular heterogeneity that underlies disease aggressiveness and tumor progression, and led to the evaluation of molecular and genetic markers associated with patients survival. The hallmark features of the tumor cell phenotype, which contribute to aggressive tumor behaviour are: its capacity for sustained proliferation, evasion of apoptosis, disregard of signals to stop proliferation and differentiation and the capacity to invade and promote angiogenesis.

Ki-67 is a nuclear antigen expressed by dividing cells. The percentage of Ki-67 expressing cells reflects the proportion of the tumor cells that are actively cycling and dividing.

Bcl-2 is an anti-apoptotic protein that is important in normal B-cell development and differentiation. The role of bcl-2 as a predictor of survival in DLBCL is controversial. Overexpression of bcl-2 protein in NHL cells has been blamed for their resistance to chemotherapy both in vitro and in vivo. Bcl-2 overexpression has been reported in approximately 40-60% of patients with DLBCL and has been associated with inferior survival. Rituximab can mitigate the negative prognostic effect of bcl-2 expression in DLBCL.

Herein, we evaluate the prognostic significance of initial bcl-2 and Ki-67 positivity in tumor cells. Furthermore we correlate these parameters with IPI and bulky disease and their significance was evaluated according to treatment response and overall survival.

Methods

Retrospective analysis was performed on 50 randomly selected patients diagnosed at the Institute of Hematology from 2001 to 2003. Patients included in the study had an initial diagnosis of DLBCL according to the World Health Organization (WHO) classification. Clinical and laboratory data from the time of initial diagnosis as well as follow up information were available. The follow-up period was up to 6 years, with median follow-up time for all patients of 40 months from the date of starting the treatment up to the last follow-up or death.

The cases with histologic transformation of indolent lymphoma into DLBCL were not included.

This study was performed on samples taken during regular clinical work-up procedures and staging after approval by the Institutional Review Board of the Institute of Hematology, Clinical Center of Serbia, according to the Helsinki Declaration and good clinical practice policy.

In all patients standard clinical and laboratory data were collected: age, gender, Ann Arbor stage, extranodal sites, bulky disease, clinical stage, B symptoms (LDH) over 5 cm. The IPI was calculated according to the five high risk features: age > 60 years, performance status > 2 (PS), Ann Arbor tumor stage 3 or 4, LDH > 460 IU/µL, and number of extranodal sites >1 while patients were divided into a low risk group (0 – 2 factors) and a high-risk group (3 or 4 factors).

This retrospective analysis was performed on 50 patients randomly selected from a large group of patients diagnosed and treated with CHOP/R-CHOP regimen with or without adjuvant radiotherapy and/or surgery over a five-year period. The response rate was determined according to standard Cheson criteria.

The 4 µm thick tissue sections were dehydrated and deparaffinized according to standard procedures and stained with haematoxylin and eosin, Giemsa and reticulin (Gordon Sweet), and examined by light microscopy.

For immunohistochemical analysis, pretreatment antigen retrieval by microwave heating in 10 mM citrate buffer pH 8 was performed according to the manufacturer directions and current laboratory protocol. Incubation was performed using the following primary antibodies: bcl-2 (clone 124, Dako, Denmark) and Ki 67 (clone MIB 1, Dako, Denmark), together with standard diagnostic panel for DLBC NHL.

Immunoreaction with a standard indirect immunoperoxidase technique was developed applying an avidin-biotin complex method (ABC, LSAB2 kit peroxidase, Dako, Denmark) using AEC as chromogen. Sections were counterstained with Mayer hematoxylin.

Two hematopathologists reviewed and evaluated all slides at the time of immunohistochemical analysis. They confirmed the diagnosis of DLBCL according to criteria outlined by the WHO classification. The percentage of tumor cells with Ki-67+ nuclear staining on 10 different high power microscopy fields (HPF, 400×) was determined. The intensity of this staining were graded as weak (0-30% Ki-67+), moderate (31-60% Ki-67+ cells), and strong (>60% Ki-67+ cells). Tumors were considered positive when at least 50% of tumor cells expressed bcl-2 protein.

The response rates were analyzed according to widely accepted international Cheson criteria. For the purpose of statistical analysis, partial remission, non response and progressive disease were considered as treatment failures. Event free survival (EFS) was measured from the start of treatment to the date of primary treatment failure, relapse, or the date...
of last follow-up. Overall survival (OS) was measured from the beginning of treatment to the time of last follow-up (censored patients) or time of death.

For univariate analysis, the $\chi^2$ and Fischer exact tests were used to assess the association between molecular and clinical and laboratory variables. Survival analysis was performed by the Kaplan-Meier method. The statistical significance of differences in EFS and OS between groups of patients was estimated by the logrank test. Statistical significance of prognostic variables was also evaluated by multivariate analysis using Cox proportional hazard model. For nonparametric variables and analysis of factors influencing outcome of treatment, nonparametric tests Mann-Whitney U test and Kruskall-Wallis test were applied.

All tests were two-sided at the threshold of $p$ values = 0.05. Values $p < 0.05$ were considered statistically significant. All statistical analysis were performed by licensed Statistical Analysis Software (Stat Soft, Inc. Tulsa USA, 2005. STATISTICA data analysis software system, version 7.1; www.statsoft.com) 16.

Results

Laboratory and clinical features of 50 patients (30 male/20 female) with DLBCL are listed in Table 1. The patients age ranged from 17–87 years, with the mean age of 50 ± 18.16 years. On presentation, 64% of patients were classified as being in stage III and stage IV. High IPI was present in 36% of patients. Initial bulky disease was confirmed in 24%, extranodal distribution in 68%, B symptoms in 76% and LDH > 460 IU/µL in 62% of patients. After the first line therapy, complete remission (CR) was achieved in 39 patients (78%), while treatment of 11 patients failed. After a 5-year follow-up, 28 (56%) patients were still in CR, while 22 (44%) patients died.

Statistical analysis of certain clinical variables revealed that patients with bulky form of the disease had significantly higher LDH levels ($t$-test and $\chi^2$, $p < 0.05$), but there was no association between bulky disease and other clinical variables.

Concerning remission rates, we were unable to show that bulky or extranodal disease, constitutional symptoms or LDH level bore any relation to CR rates ($\chi^2$, $p > 0.05$).

According to IPI, the distribution of patients was as follows: IPI ≤ 1 in 11, IPI ≥3 in 18 patients, meaning that low IPI score was present in 32 and high IPI score in 18 patients. Concerning IPI score values we found no correlations between low and high IPI and bulky disease. Univariate survival analysis showed that high IPI had statistically significant negative influence on OS, and also on EFS (logrank, $p < 0.01$). Patients with IPI ≥3 had significantly more progressive disease and shorter overall survival (OS5y not reached, median OS 7 months compared to OS5y of 87% in patients with low IPI).

Univariate analysis showed that extra nodal disease, bulky form, and the existence of B symptoms had certain influence on OS and EFS but these differences were not statistically significant (log rank, $p > 0.05$), whereas increased LDH level did have a significant effect on overall survival.

Multivariate analysis revealed that IPI > 3 had an independent prognostic impact on survival ($p < 0.01$) together with extranodal distribution and LDH levels ($p < 0.05$) as a part of IPI score.

The cell proliferation marker Ki-67 was observed as nuclear staining in tumor cells and in lymphocytes within the tumor tissues in all 50 cases. The staining intensity and number of tumor cells positive to Ki-67 varied from case to case, ranging from 30% to 95%. None of the patients had more than 95% Ki-67 positive tumor cells.

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Table 1
Statistical analysis of clinical variables revealed that there was a correlation between high Ki-67+ and high LDH levels, but this was not statistically significant (t-test and χ² p > 0.05).

Values of Ki-67 positive tumor cells follows the values of IPI scores, meaning that patients with a high proliferation rate also had high IPI value (IPI > 3). We found statistically significant positive correlation between low and high Ki-67+ and IPI scores (χ², Mann Whitney U test and Spearman Rank correlation, p < 0.05).

Thirty of 50 patients (60%) had Ki 67+ in > 60% of tumor cells. In the group with fatal outcome 15 out of 22 patients had Ki 67+ in > 60% of tumor cells (Figure 1). The logrank test analysis signified that patients with a high proliferative fraction (Ki-67 > 60%) had a worse overall survival (OS5y) of 40% compared to those with low proliferation (Ki-67 < 60%) with OS5y of 80% (p < 0.01) (Figure 2). In multivariate analysis performed by Cox model we found that high Ki-67+ (at a threshold of 60% of positive cells) was marginally significant (p = 0.057).

Antiangiogenic protein bcl-2, was detected as a distinct cytoplasmic staining pattern of the tumor cells. Immunohistochemical analysis revealed that bcl-2+ was dominantly high (> 50%) in 21 patients (42% of analyzed cohort) (Figure 1). Furthermore these patients had significantly shorter OS (p < 0.05).

Concerning bcl-2 positivity at a threshold level of 50% of positive labeled cells, we found the same pattern as was detected for Ki-67, meaning that patients with high percentage of bcl-2+ tumor cells also had high IPI value (IPI > 3) (Figure 1), and there was statistically significant positive correlation between low and high bcl-2 and IPI scores (χ² Mann Whitney U test and Spearman Rank correlation, p < 0.05).

There was a negative correlation between bcl-2+ and achievement of CR (66% vs. 86% in patients with bcl-2 high and low levels respectively, p < 0.05). Patients with low bcl-2 expression (< 50% of tumor cells) survived significantly longer (OS5y median time rate 68%) compared to those with high bcl-2+ (OS5y 37%) (Figure 3). In the study of Hans et al 9 the IPI score predicted OS (p < 0.001) and EFS (p < 0.001) when comparing those with low (0-2) versus high (3-5) scores. In our study, univariate survival analysis showed that high IPI had a statistically significant negative influence on overall and also on event free survival time (logrank, p < 0.01). The patients with IPI ≥ 3 had significantly more progressive disease and shorter overall survival (OS5y not reached, median OS 7 months compared to OS5y of 87% in patients with low IPI). Multivariate analysis revealed that IPI > 3 had an independent prognostic impact on survival (p < 0.01) together with extranodal distribution and LDH levels (p < 0.05). In the study of Colomo et al. 17 the IPI

Discussion

Although studies of individual biomarkers have improved our understanding of the pathogenesis of DLBCL, many studies have yielded conflicting results. Reasons for these discrepancies include the retrospective nature of most studies, small patient sample size, lack of uniformity in technique and failure to control other simultaneous biologic processes that may confound outcomes. Importantly, these markers need to be revalidated in patients who have been treated with immunomodulatory therapy 4.

In the study of Hans et al 9 the IPI score predicted OS (p < 0.001) and EFS (p < 0.001) when comparing those with low (0-2) versus high (3-5) scores. In our study, univariate survival analysis showed that high IPI had a statistically significant negative influence on overall and also on event free survival time (logrank, p < 0.01). The patients with IPI ≥ 3 had significantly more progressive disease and shorter overall survival (OS5y not reached, median OS 7 months compared to OS5y of 87% in patients with low IPI). Multivariate analysis revealed that IPI > 3 had an independent prognostic impact on survival (p < 0.01) together with extranodal distribution and LDH levels (p < 0.05). In the study of Colomo et al. 17 the IPI

also had a high value for predicting OS (p<0.00001). Extranodal disease, bulky disease, and B symptoms had certain influence on OS and EFS, but these differences were not statistically significant (logrank, p>0.05). Increased LDH level had significant effect on overall survival.

Diffuse large B cell lymphoma is heterogenous, and the expression of bcl-2 is variable 18.

In most studies, bcl-2 protein expression alone or in combination with other factors (e.g. high IPI) is regarded as evidence of both 1 (14;18) and an aggressive clinical course, the OS and disease-free survival curves being significantly worse than in bcl-2- cases (19–22). In the study of Colomo et al. 17 bcl-2 expression was included in the Cox model along with the IPI (low risk vs low/intermediate-risk vs high/intermediate-risk vs high-risk). Bcl-2 maintained a trend of independent prognostic value, although the IPI remained most significant variable.

Concerning bcl-2 positivity at the threshold level of 50% of positive tumor cells, we have found that patients with high percentage of bcl-2+ tumor cells also had high IPI value (IPI > 3), and there was statistically significant positive correlation between low and high bcl-2 and IPI scores (χ², Mann Whitney U test and Spearman Rank correlation, p < 0.05). Furthermore, there was a clear difference between bcl-2+ and the achievement of CR (66% vs 86% in patients with bcl-2 high and low levels respectively, p < 0.05). Multivariate analysis by Cox regression model revealed that bcl-2 positivity (threshold 50%) had a significant independent prognostic value concerning OS (p < 0.05).

However, multiple studies have looked at the expression of bcl-2 using immunostains, and most have found no difference in OS (23–25).

A critical look at the various results during the last 10 years shows considerable variation. Although a predictive value for DFS is reported in most series, the predictive value of bcl-2 positivity for OS is not reliably consistent among those reports. Although some series may lack the statistical power to detect survival difference, variations in scoring and interpreting bcl-2 staining are likely to account for such discrepancies 26.

The prognostic significance of Ki-67 expression in DLBCL is controversial 6. Tumors with low Ki-67 index may exhibit resistance to chemotherapy, given that the majority of the malignant cells are in G0/G1 and thus are resistant to cycle specific cytotoxic chemotherapy. Furthermore, G0/G1 arrested cells have time to repair DNA damage induced by the chemotherapy and thus survive 6. The proliferative fraction in DLBCL as detected by Ki-67 staining is usually high (> 40%) and may be greater than 90% in some cases 27.

Miller et al. 28 analyzed the prognostic significance of Ki-67 staining in 60 representative DLBCL patients from the Intergroup 0067 study that compared four different anthracycline-based regimens, and found that the 3-year OS was significantly shorter in patients with Ki-67 nuclear expression in 80% or more malignant cells. In a subsequent study on 105 DLBCL patients, the same authors demonstrated that high proliferative activity, defined in this study as nuclear Ki-67 expression in greater than 60% of malignant cells, was a strong predictor of poor survival (logrank, p = 0.003, )27. The Nordic Lymphoma Group Study defined low expression of Ki-67 as less than 60% of tumor cells, and found that expression of Ki-67 was not associated with significant differences in a 5-year OS 29.

We found that values of Ki-67 positive tumor cells follows the values of IPI scores, meaning that patients with high proliferation rate also had high IPI value (IPI > 3). There was a statistically significant positive correlation between low and high Ki-67+ and IPI scores (χ², Mann Whitney U test and Spearman Rank correlation, p < 0.05). The logrank test analysis signified that patients with a high proliferative fraction (Ki67 > 60%) had worse overall survival (OS5y) of 40% compared to those with low proliferation (Ki67 < 60%) with OS5y of 80% (p < 0.01) (Figure 2). In multivariate analysis performed by Cox model we found that high Ki-67+ (at a threshold of 60% of positive cells) was marginally significant (p = 0.057).

Conclusion

We found that a high percentage of bcl-2 and Ki-67 positive tumor cells was associated with worse prognosis. Furthermore there was positive correlation between these molecular parameters and high IPI score.

Taken together, biological factors in combination with clinical models such as IPI, might become an integral part of our daily practice in finding a way to a more individualized or patient-tailored practice in oncology.

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