ACTA MEDICA SALINIANA

ORIGINAL

PROGNOSTIC SIGNIFICANCE OF BCL-6 PROTEIN AND KI-67 MONOCLONAL ANTIBODY IN LARGE CELL B-LYMPHOMA TREATED WITH POLICHEMIOTHERAPY WITH OR WITHOUT ADDITION OF RITUXIMAB

Aida Arnautovic-Custovic[1], Elmir Cičkusic[2], Samira Hasic[1], Haris Sahovic[1], Predrag Jovanovic[3], Svetlana Jovic[4]

ISSN 0350-364X

Aida Arnautovic-Custovic[1], Elmir Cickusic[2], Samira Hasić[1], Haris Sahovic[1], Predrag Jovanovic[3], Svetlana Jovic[4]

DOI: 10.5457/ams.165.10

Introduction: Diffuse large cell lymphoma (DLCL) is the most common type of non-Hodgkin lymphoma (NHL), accounting for almost 35-40% of all cases of NHL.

Aim: To analyse the expression of Ki-67 i bcl-6 in large cell lymphoma tissue, treated with CHOP and CHOP+R protocol, and to evaluate the level of therapeutic response and the duration of progression free survival in patients treated by therapeutic protocols CHOP and CHOP+R.

Methods: For analysis of both Ki-67 and bcl-6 we used criterion for positivity of more than 10% protein expression in lymphoid tissue.

Results: In total sample there was 48.3% bcl-6 positive cells. Comparing two different treatments, there was no difference in level of bcl-6 expression. (Mann-Whitney; U=398; p=0.44). No significant difference was found in incidence of bcl-6 positive patients between two types of treatment (X2=0.60; df=1; p=0.44). All the patients were Ki-67 positive. Compared within two therapeutic groups, there was no difference in level of Ki-67 expression (Mann-Whitney; U=403.5; p=0.49). In the group of patients treated with CHOP protocol niether bcl-6 nor Ki-67 showed influence on duration of progression free survival. In the group of patients treated with CHOP+R protocol, there was also no influence of bcl-6 and Ki-67 on duration of progression free survival.

Conclusions: Results suggest that Ki-67 and bcl-6 expression in tumor tissue can not be used as indicators for the level of therapy response and progression-free survival in DLCL treated with polichemiotherapy with or without addition of Rituximab.

Keywords: diffuse large cell lymphoma, bcl6, Ki-67.

Institutions:
1 Clinic for Oncology, Hematology and Radiotherapy, Department of Hematology, University Clinical Center Tuzla, Tuzla
2 Policlinic for Laboratory Diagnostics, University Clinical Center Tuzla, Tuzla
3 Clinic for Internal Diseases, Department of Gastroenterology, University Clinical Center Tuzla, Tuzla
4 Policlinic for Blood Transfusion, University Clinical Center Tuzla, Tuzla

Received:
28.09.2010
Accepted:
22.11.2011

Correspondence:
Aida Arnautović Čustović1 Department of Hematology, Clinic for Oncology, Hematology and Radiotherapy, JZU UKC Tuzla, 75000 Tuzla, Trnovac b.b., Bosnia and Herzegovina
Tel: +387611933000

mail: aidahem@yahoo.com

Competing interests
The authors declare no competing interests.

INTRODUCTION

Diffuse large cell lymphoma (DLCL) is the most common type of non-Hodgkin lymphoma, accounting for almost 35-40% of all non-Hodgkin lymphoma. It is most frequently diagnosed in elderly patients, over 65 years of age and makes almost one half of all lymphoma diagnosed in that life period.1

In clinical practice numerous prognostic parameters are used to define the outcome of the treatment, whose significance still remains unrecognised. It is especially important to emphasize the fact that the significance of already accepted prognostic parameters alters constantly as a result of introducing new modalities of treatment, which results in lack of clinical significance of those parameters.

Germinal center phenotypes (GC) and non-germinal center phenotypes (non-GC) are the predictors of outcome of diffuse B-cell lymphoma and can be used to differentiate the patients of high and low risk.

Various studies attempted to identify the phenotype of the germinal and non-germinal center using markers such as bcl-6, CD10, Ki-67, MUM-1 and CD138. Generally speaking, the germinal center phenotype, especially including bcl-6 expression, is connected with more favourable prognosis of disease.2,3

BCL-6 is a protooncogen involved in DLBCL pathogenesis. Reverse transcriptase PCR (polimerase chain reaction) in real time was introduced in practice in order to ensure accuracy and evaluate the expression of m-RNA of bcl-6 proteins. Bcl-6 mRNA expression was observed in tumor samples of patients with DLBCL.

The expression of bcl-6 protein, marker of genesis of GC neoplasm, is associated with more favourable outcome of DLBCL.4

The growth fraction of the tumor in patients with NHL showed correlation with the survival period in retrospective studies. The growth fraction of the tumor can be evaluated by IHH tecnhiques that use Ki-67 monoclonal antibody (MoAb), which marks the nuclear protein material in mytotic cells. The expression of Ki-67 MoAb is associated with malignant prognosis of DLBCL. It is neccessary to provide secure treatment

of diagnostic material and interpretation of results by an experienced hematologist. The use of an adequate thin needle biopsy should preserve the architecture of the lymph node.

Algorithm for treating advanced relapsed/refractory form of DLBCL is as follows: Rituximab+CHOP 21 should be viewed as a standard treatment for patients with advanced stadium of DLBCL, no matter to which risk group they belong to. The role of support therapy after induction with Rituximab remains unclear.

The aim of our study was to analyse the expression of Ki-67 and bcl-6, in large cell B lymphoma tissue, in patients treated with CHOP and CHOP+R protocol, and to determine the level of therapeutic response and duration of progression free survival in patients with large cell B lymphoma, treated with therapeutic protocols CHOP and CHOP+R, and the influence of Ki-67 and bcl-6 expression.

PATIENTS AND METHODS

Investigation was retrospective-prospective using consecutive sample of 60 patients who were diagnosed with DLBCL patohistologically and immunohistochemically, and who were afterwards treated with therapeutic protocol CHOP (n=30) and CHOP+R (n=30). The study included patients both male and female, regardless of the age, hospitalized in JZU University Clinical Center Tuzla, from 2003 to 2009.

For analysis of Ki-67 and bcl-6 expression, archived samples of lymphoma tissue were used from the Department of Pathology UKC Tuzla. All the samples were previously fixed in 10% formalin solution and inserted into parrafin. Histological slices with thickness of 4 μm were after deparaffinization and blocking the endogenous peroxidase, treated in citrate buffer (10 mM pH 6.0) on 100°C in duration of 15 minutes. In the center for immune staining Sequenzy-Shanadon, all levels of incubation were performed. Following the preincubation period with 100% normal bovine serum, a three level immunoperoxidase treatment was performed. Primary antibodies Ki-67 (MIB) and bcl-6 (PG-B6p) in solution 1:20 were used (DAKO, Glostrup, Denmark).

Positive reaction was defined as clear brown staining of the lymphoma cells nuclei, with no unspecific staining of the enviroment. Positive result was considered as the expression of Ki-67 and bcl-6 in nuclei in more than 10% lymphoma cells, while the level of expression was determined in per cents after analysis of 500 tumor nuclei.

Treatment of large cell B lymphoma was conducted with standardized therapeutic protocol CHOP and CHOP+R.5,6,7,8 Patients were continuously followed from 2003 to the end of 2009. The evaluation of therapeutic response was done after total administration of therapy, based on physical examination, level of serum LDH, ultrasound examination and computerized tomography. The therapeutic response was defined as complete remission, if the previously mentioned methods could not detect the presence of the tumor, partial response, if there was a residual tumor present after complete treatment, and absence of response if the tumor/stadium increased after complete treatment, and finally progression of disease if the tumor/stadium increased in spite of completed therapy. Progression free survival was defined as a period during which the disease made no progression, from the moment of posttherapeutic evaluation untill the next evaluation in which progression of the disease was determined.

All data were analysed using statistical package SPSS 18.0 (SPSS Inc, Chichago, IL, USA). Standard tests of descriptive statisitics were done (central tendency and dispersion). All variables were tested according to central distribution using Kolomogorov-Smirnov test. Quantitative variables were tested using Students t-test if they were normally distributed, and Mann-Whitney test, if they were distributed assimetrically. Qualitative variables were tested using hi-square test. Significant correlation between variables was tested using non-parametric Spearman test.

The evaluation of the progression free survival was done using Kaplan-Meier analysis, while testing the influence of individual factors on the duration of progression free survival was done using Cox regression analysis. All tests were done with the level of significance 95% ($p<0.05$).

RESULTS

The median of procentual expression of bcl-6 in total sample was 15.5% with interquartile range of 5.5% to 54.5%, minimum 0% and maximum 99%. Considering the criterion of positivity of bcl-6 of 10% positive lymphoma cells, in total sample there was 29/50 (48.3%) positive to bcl-6.

Comparing the two treatments, there was no difference in level of expression of bcl-6 (Mann-Whitney; U=398; p=0.44) –Figure 1. There was also no significant difference in incidence of bcl-6 positive patients (considering the criterion of more than 10% expression in lymphoma cells) between two types of treatment (X2=0.60; df=1; p=0.44) – Table 1.

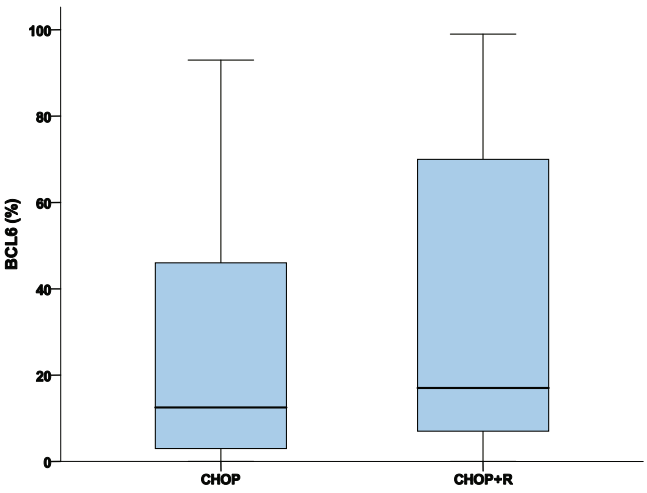

Figure 1. Comparison of bcl-6 expression between two therapeutic groups before the treatment

Table 1. Procentual distribution of bcl-6 expression in both therapeutic groups

Type of treatment (%)	bcl-6 positive (%)	bcl-6 negative (%)
CHOP	43.3	56.7
CHOP+R	53.3	46.7

The median of procentual expression of Ki-67 in total sample was 59% with interquartile range of 33% to 74%, and minimum of 11% to 96%. All the patients were Ki-67 positive, taking into account the criterion of more than 10% positive lymphoma cells. Comparison between two therapeutic groups showed no difference in the level of Ki-67 expression (Mann-Whitney; U=403.5; p=0.49) – Figure 2.

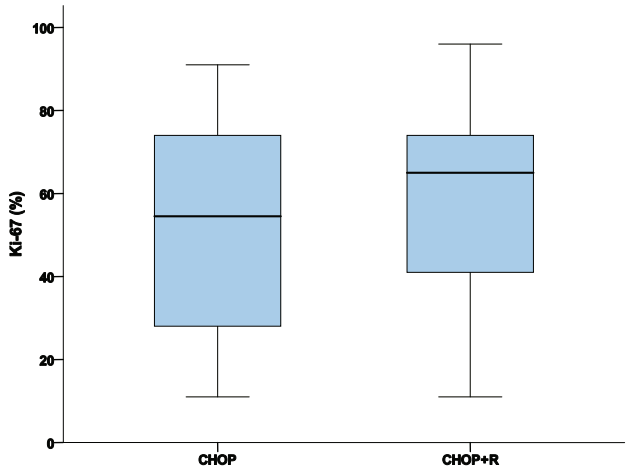

Figure 2. The level of expression of Ki-67 in both therapeutic groups

Separate Cox analysis for both groups of treated patients were done in order to investigate prognostic influence of bcl-6 and Ki-67 on duration of progression free survival.

In the group of patients treated with CHOP protocol niether the level of expression of bcl-6 (OR=0.99; %CI=0.96-1.02; p=0.44) nor Ki-67 (OR=0.99; %CI=0.97-1.02; p=0.60) showed significant influence on duration of progression free survival.

In the group of patients treated with CHOP+R protocol, there was also no significant bcl-6 expression (OR=0.93; %CI=0.82-1.07; p=0.32) nor Ki-67 expression (OR=1.02; %CI=0.97-1.08; p=0.42) that influenced the duration of progression free survival – Figure 3.

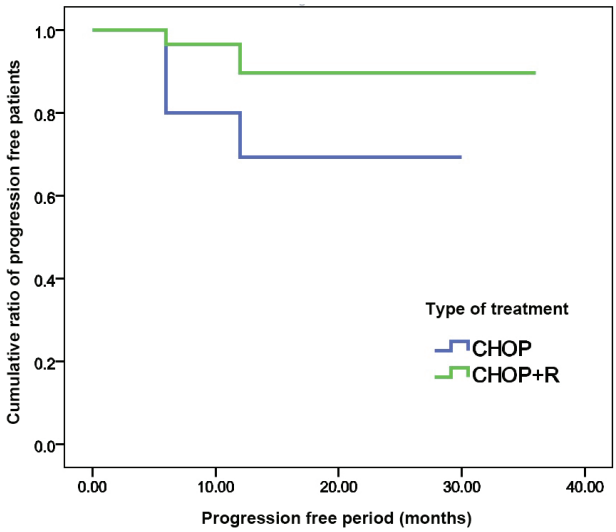

Figure 3. Duration of progression free period in both therapeutic groups

DISCUSSION

Reffering to the WHO classification, DLBCL is defined as a mature B-cells neoplasm. Imunophenotypically tumor cells show markers on B-cells CD19, CD20, CD22, CD97a as well as CD45, monoclonal surface IgM antigen. In rare cases of DLBCL, cells show markers CD10+ and CD5+. A few morphological variants of DLBCL posses characteristic CD45 receptors on their surface along with pan-B-cell marker CD20 and CD79a, based on morphological appearence of cells, cell enviroment and immunophenotype. The combination of staining techniques for CD10, bcl-6 and MUM-1 have been used as an immunocytochemical surrogate for identification of germinal center phenotype (CD+ or CD10-, bcl-6+ or MUM1-) as opposed to non-germinal center DLBCL.5,6

The expression of bcl-2 protein is evident in 30-50% patients. A proportion of cases express CD5, but these are negative for cyclin D-1. Staining for CD30 is seen in some cases (up to 16% in some series). Expression of CD138 may be seen in cases showing plasmablastic morphology, but is rarely seen in other cases.

DLBCL imunnophenotypically has high expression of B-cells markers, especially CD20, then CD19, CD22 and CD79a. HLA-DR is the most frequent activation antigen, while the expresion of CD23 is rarely found. One third of patients has CD5 and CD10 which is a sign of transformation of small cell lymphoma or follicular lymphoma to DLBCL.

Numerous variants of DLBCL are discribed in terms of morpfological and immunohistochemical characteristics. WHO classification divides DLBCL in mediastinal or thymic, intravascular and primary effusion lymphoma (PEL). Cytogenetic and mollecular analysis provide additional insights in DLBCL pathogenesis. For example, rearangement of bcl-2 is seen in 20-30% of patients and is associated with the less favourable prognosis. Similar to that, bcl-6 gene, which is known to

have structural similarities with the class of transcriptional factors included in control, proliferation and differentiation of cells, is often found in some subtypes of DLBCL and correlates with better income of disease.

Alizedah at al. published in the year 2000 the aplication of complementary DNA (cDNA) microarray technique for diagnosis of DLBCL, showing that the diversity in gene expression in DLBCL reflects the level of proliferation, patient response to therapy and state of differentiation of the tumor. After that, international group of scientists connected these results by reviewing the gene expression of DLBCL in 240 patients, using microarray technique. Three subgroups were identified with the clear gene expression: germinal center type (GC), activated B-cell and diffuse large cell B lymphoma. Two very important moments were observed: bcl-2 translocation and C-REL amplification were only detected in subgroup GC, which showed the best 5-year survival period.7

Shipp et al. showed that the result of treatment of DLBCL with standard PHT was evidently dependent on gene expression which regulated the B-cell response to signalling, critical serin/treonin phosphorilation paths and apoptosis. Recent study of Stanford University tried to identifie the level of expression of 6 genes: LMO-2, bcl-6, CCND-2, SCYA-3, BCL-2 and Ki-67 using PCR technique, which was sufficient to predict the overall survival in patients with DLBCL.8

The results of evaluating the prognostic markers of DLBCL are widely investigated. Apart from biological variations, this can cause differences in laboratory techniques being used. International cooperation in clinical research of lymphoma is based on validation and standardization of immunohistochemical techniques for identifiing some currently most popular prognostic markers of DLBCL. DLBCL is characterized with significant level of morphological and clinical heterogeneity. Parameters have been established to predict the outcome of treatment and help in identification of patients who can benefit from therapy, which is adapted to the risk-group to which they belong.

Jane N. Winter et al. made a prospective study in 2000 with the aim to evaluate the prognostic significance of bcl-6 protein in patients who were treated with Rituximab added to PHT (CHOP).9 In phase three of the study which compared R/CHOP with CHOP protocol 199 patients were included. Bcl-6 negative patients treated with R/CHOP protocol showed longer failure free survival (FFS) an overall survival (OS) compared to patients treated with CHOP protocol only (2-year FFS 76% to 9% and OS 79% to 17%). As a contradiction to that there was a clear evidence of no difference in FFS and OS in the observed group of patients with positive bcl-6 protein. The only significant predictor of the treatment outcome was IPI.

In the year 2007 a group of authors examined the way the combination of Rituximab with PHT influenced the clinical income of patients with DLBCL with different phenotypes. GC anf non-GC phenotypes were immunohistochemically identified from the samples of 90 de novo diagnosed patients. These patients were treated with the combined protocole R/CHOP. 104 patient were previosly treated with PHT CHOP alone and they were used as a control group. In correlation with the previous studies, patients treated with PHT with IHH defined GC phenotype, showed significant increase of OS and FFS compared to non-GC group of patients (OS 70% to 47%, FFS 59% to 30%). In contradiction to that, IHH defined GC phenotype was not the predictor of the treatment outcome in patients treated by immunochemotherapy (OS 77% to 76%, FFS 68% to 63%). Finally, it was concluded that Rituximab combined with PHT eliminates the prognostic significance of immunohistochemically defined GC and non-GC phenotype in patients with DLBCL.

The growth fraction of tumor in patients with NHL showed correlation with survival in retrospective studies. The growth fraction can be evaluated by IHH techniques which use Ki-67 monoclonal antibody (MoAb), which marks the nuclear protein material in mytotic cells. TP Miller et al. made a study in 1994 with the aim to evaluate the clinical benefit of Ki-67 MoAb in prognosis of disease outcome.10 Applying the design of prospective study in multiinstittutional cooperative study groups, proliferative index, clinical outcome and statistical correlations were made independently for patients with no previous treatment. The relation between Ki-67 positive cells was determined using method of snap frozen thin tissue section. The proliferative index was 80% and more, as determined in previous retrospective studies. It identified the group of patients with highly negative Ki-67 associated proliferation index in comparison with the patients who had low proliferation index. One-year overall survival (OS) was 82% (low proliferation index) to 18% (high proliferation index). Multivariant analysis of disease regression which contained commonly used factors of clinical prognosis, confirmed the independent effect of proliferation on the period of survival. It was concluded that Ki-67 MoAb identifies the group of patients with agressive NHL, for whom the existing therapeutic protocoles are not adequate.

Taking into account different clinical and biological parameters in patients with large cell B lymphoma, divided into different subgroups with different prognostic factors, individual approach was concluded necessary in treatment of these patients. CHOP (cyclophosphamid, adriamicin, oncovin, prednison) protocol combined with immunotherapy represents gold standard for treatment of DLBCL. In the middle 70s of the last century the appearence and development of genetic ingineering produced monoclonal antibodies for treatment of large cell B lymphoma. Introduction of monoclonal antibodies that bind to CD20 antigens in therapy of DLBCL, so- called anti-CD20 antibodies started in 1980. The results of the first randomized study III phase GELA study (Group d'Etude des Lymphomes de l'Adulte)10 which included 399 previously untreated patients age 60-80 years with DLBCL, randomized to receive VIII cycles of PHT CHOP protocol for 21 days or the same therapy with the addition of Rituximab (R+CHOP), which was administered on the first day of every cycle, show that the complete remission in patients with combined therapy (CHOP+R) was 77%, compared with 64% in patients treated with CHOP protocol only. Two year free survival for patients

treated with CHOP+R was 73%, and for patients treated with CHOP only it was 61%.11

Another study, published by Pfeundschuh et al. in 2008, showed that patients age 18-61, treated with CHOP+R, achieved complete remission in 81 cases, while patients treated with CHOP only achieved complete remission in 67% cases.12 Anti-CD20 antibodies, after binding to transmembranic CD20 antigen on B-lymphocytes, react in two ways: they induce antibody dependent cell mediated cytotoxicity or complement dependent cytotoxicity, or in other words they initiate apoptosis (programmed cell death). Anti-CD20 antibodies inhibit the proliferation of lymphoma cells, and they make them more sensitive to cytotherapy. Addition of antiCD-20 antibodies to the standard CHOP protocol in patients with DLBCL gives a possibility of achieveing longer 5-year survival. This was acknowledged with results of many studies that compared achieved results in patients treated with CHOP and CHOP+R protocol.

The addition of Rituximab to CHOP protocol in elderly patients was investigated in the USA, including patients over the age of 60 with previously untreated advanced DLBCL. Patients were randomized in groups that were treated with VI-VIII cycles of CHOP protocol or CHOP combined with Rituximab. Second randomization reffered to patients that were only observed and second group of patients who recieved support therapy with Rituximab once a week, in duration of 4 weeks in intervals of 6 months. Total of 632 patients initially treated with addition of Rituximab were divided to observation or support therapy with Rituximab. There was significant difference in 3-year failure free survival-FFS in the group that recieved R+CHOP in comparison with the group of patients that were treated with CHOP protocol only (53% to 46%) The advantage of aplication of supporting therapy with Rituximab was obviously limited to patients who did not recieve this medicine as a first line therapy. There was no significant difference in overall survival between these two groups, due to the fact that 40% patients in the group that received CHOP protocol only, also recieved Rituximab in the second randomization.13

Other results that show benefit of Rituximab were published in retrospective study from British Columbia, Canada, which showed longer FFS and OS in younger low risk patients who were treated with Rituximab. The benefit of Rituximab in younger low risk patients was shown in MinT (Mabthera Inernational Trial).14 This study included patients with DLBCL „bulky disease“ I to II-IV stadium, age 18-60 years with IPI score 0 and 1. Treatment included VI cycles of CHOP, CHOEP or similar PHT regimes with or without Rituximab, which was administered on the first day of treatment in 326 randomized patients. Time to treat failure (TTF) was 76% for patients treated with PHT combined with Rituximab in comparison with 60% for patients treated with PHT only. Overall survival was 94% to 87%. These results should conclude that addition of Rituximab had benefit in all risk groups. Further analysis should show that the expresssion of byological markers such as bcl-2 protein could differentiate patients who could benefit from adding Rituximab to their classic PHT.

There is no clear evidence that supports the usage of high dose therapy and stem cells transplantation as a first line therapy, even for patients with low risk. This approach should be further investigated in prospective studies.

The median of bcl-6 expression in total sample was 15,5% with range 5,5 to 54,5%, minimum 0% and maximum 99%. Considering the criterion for bcl-6 positivity of 10% positive lymphoma cells, in total sample there were 29/60 (48.3%) cells positive to bcl-6. The comparison between the two groups of patients treated with different protocols showed no difference in level of bcl-6 expression, nor in incidence of bcl-6 positive patients in these two groups. Patients treated with CHOP protocol had 43.3% bcl-6 positivity, and 56.7% negative bcl-6 expression. Patients treated with protocol CHOP+R had 53.3% bcl-6 positivity and 46.7% showed no positivity.

The median value of procentual Ki-67 expression in total sample was 59% with interquartile range of 33% to 74%, and minimum 11% to 96%. All the patients were Ki-67 positive, considering the criterion of more than 10% expression in lymphoid tissue. Compared within two therapeutic groups, there was no difference in level of Ki-67 expression. In the group of patients treated with CHOP protocol niether bcl-6 nor Ki-67 had no influence on duration of progression free survival. In the group of patients treated with CHOP+R protocol, there was also no significant influence of bcl-6 nor Ki-67 expression on duration of progression free survival period.

LITERATURE

1. Anonymous. The Non Hodgkins Lymphoma Classification Project. A Clinical Evaluation of the International Lymphoma Stady Classification of Non Hodgkins Lymphoma. Blood 1997; 89: 3909-3918.
2. Anonymous. Facts 2006-2007. Fighting Blood Cancers. Surveillance, Epidemiology and The Results (SEER) Program, 2006.
3. Armitage JO. Staging Non-Hodgkin Lymphoma. Cancer J Clin 2005; 55: 368-376.
4. Lossos IS, Jones C, Warnkle 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.
5. Sehn LH, Chuczman MS et al. Optimal Use of Prognostic Factors in Non-Hodgkin Lymphoma. American Society of Haematology 2006.
6. Lennert K, Collins R, Lukes R. Concordance of the Kiel and Lukes-Collins classification of non-Hodgkins lymphomas. Histopathology 1983; 7: 549.
7. Alizadeh AA, Michael BE, Davis RE et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000; 403: 503-511.
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. Winter JN, Weller EA, Horning SJ et al. Prognostic significance of bcl-6 protein expresion in DLBCL treated with CHOP or R-CHOP. A prospective correlative study. Blood 2006: 107(11): 4207-4213.

10. Miller TP et al. Prognostic significance of the Ki-67-asociated proliferative antigen in aggressive non-Hodgkins lymphomas: a prospective Soutwest Oncology Group Trial. Blood 1994; 83: 1460-1466.

11. Coiffier B, Feugier P, Mounier N et al. Long-term results of the GELA study comparing R-CHOP and CHOP chemotherapy in older patients with diffuse large B-cell lymphoma show good survival in poor-risk patients. J Clin Oncol ASCO Annual Meeting Proceedings (Post-Meeting Edition) 25(18S, June 20 Supplement) 2007; Abstract 8009.

12. Pfreundschuh M, Shubert J, Ziepert M et al. Six versus eight cycles of bi-weekly CHOP-14 with or without Rituximab in elderly patients with aggressive CD+ B-cell lymphomas. Lancet Oncol 2008; 9(2): 105-116.

13. Coiffier B, Lepage E, Briere J et al. CHOP chemoterapy plus Rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl Med 2002; 346: 235-242.

14. Sehn LH, Donaldson J, Chhanabhai M et al. Introduction of combined CHOP plus Rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. J Clin Oncol 2005; 23: 5027-5033.