Switching to BCL-6 Negativity in Relapsed Diffuse Large B Cell Lymphoma Correlated with More Aggressive Disease Course

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Abstract Diffuse large B-cell lymphoma (DLBCL) is the most frequent, complex and heterogeneous lymphoma of adulthood. Heterogeneity is expressed at clinical, genetic, and molecular levels. It is known that BCL-6 expression is a favorable prognostic factor in DLBCL. However, the underlying mechanisms of BCL-6 expression in DLBCL relapse are not yet elucidated. Here, we present so far undescribed clinical phenomenon of switching BCL-6⁺ protein expression into BCL-6[−] expression in 19 of 41 relapsed DLBCL patients. The switch in relapsed DLBCL was associated with more aggressive clinical course of the disease. Bone marrow infiltration and high IPI risk were more often present in BCL-6[−] patients. Significantly increased biochemical parameters, such as LDH, beta-2 macroglobulin, CRP, and ferritin have been found, as well as significantly decreased serum Fe, TIBC, and hemoglobin. A Ki-67 proliferation marker was considerably high in relapsed DLBCL, but without significant differences between BCL-6⁺ and BCL-6[−] groups of patients.

Thus, switching of the positive into negative BCL-6 expression during DLBCL relapse could be used as a prognostic factor and a valuable criterion for treatment decision.

Keywords DLBCL · Relapse · Prognosis · BCL6 switching

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent subtype of non-Hodgkin lymphoma's (NHL) with frequent relapses after standard first-line therapy [1, 2]. The clinical outcome reflects cytogenetic and molecular heterogeneity within DLBCL where BCL-6 gene rearrangement is the most common chromosomal abnormality [3].

The BCL-6 proto-oncogene is essential for normal germinal center (GC) formation and important element in lymphomagenesis [4]. Hierarchical clustering of the gene expression identified two molecularly distinct forms of DLBCL. One is GC-like (GCB) DLBCL, derived from different genetic aberrations, such as t(14;18) translocation involving BCL2, amplification of the mir-17–92 microRNA in the MIHG1 locus cluster on chromosome 13, gain/amplification of a 7.6-Mb region on chromosome 12, deletion of the PTEN tumor suppressor gene on chromosome 10, and amplification of the REL locus on chromosome 2. The other type is the activated B cell-like (ABC) DLBCL subtype, derived from aberrations such as trisomy 3, deletion of chromosome arm 6q, gain/amplification of a region on chromosome arm 18q, deletion of the INK4a/ARF tumor suppressor locus on chromosome 9, and gain/amplification of a 9-Mb region on chromosome 19 [5]. These findings entirely elucidated molecular basis of previously defined immunohistochemical patterns of GCB

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DLBCL (BCL-6⁺, CD10⁺, MUM-1[−]) and ABC DLBCL (BCL-6[−], CD10[−], MUM-1⁺) type [6].

The B cells and CD41⁺ T cells in the GC express BCL-6, which controls GC formation [7]. This expression can be affected by chromosomal translocations in the DLBCL patients [8]. Thus, BCL-6 expression could predict survival in patients with DLBCL [8, 9].

Still, BCL-6 expression is not routinely used prognostic marker and it is not incorporated in prognostic indexes yet, as well as none of the other biomarkers. In routine clinical practice, the International Prognostic Index (IPI) consisted of clinical stage, patient's age, LDH level, extranodal involvement and Eastern Cooperative Oncology Group performance status (ECOG PS), is used as the tool for risk stratification and treatment tailoring [10].

The aim of this study was to evaluate biochemical and clinical characteristics, as well as immunohistochemical findings in two groups (BCL-6⁺ vs. BCL-6[−]) of relapsed DLBCL patients—who were all BCL-6⁺ at diagnosis. We proposed that switching of positive to negative BCL-6 expression could be a triggering event and prognostic factor for more aggressive course of DLBCL at relapse.

Materials and Methods

We investigated 41 patients with histologically confirmed relapse of DLBCL at the Clinic for Hematology since 2005 until 2010. They were all BCL-6⁺ at diagnosis due to their GCB immunohistochemical pattern. All the patients achieved complete remission on first-line therapy and relapsed at least 3 months after the end of initial treatment. Among biochemical and clinical parameters we evaluated: lactate dehydrogenase (LDH), beta-2 microglobulin (b2M), C-reactive protein (CRP), ferritin, iron (Fe), total iron binding capacity (TIBC), hemoglobin, MCV, hematocrit, uric acid and IPI based on clinical stage, LDH, patient's age, extranodal involvement and ECOG PS.

All patients were initially treated with R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) and radiotherapy on the site of bulk tumor if it was present on diagnosis. In the first relapse they received salvage chemotherapy according to ESHAP (etoposide, methylprednisolone, cytarabine, cisplatin) regimen. In the second relapse patients received chemotherapy according to MINE regimen (ifosfamide, mitoxantrone, etoposide).

Immunohistochemical studies were performed on 4 µm sections from paraffin-embedded tissue. Histology slides were stained by hematoxylin-eosin (HE) and Giemsa in the tumor specimens. Immunostaining was performed using a labeled streptavidin-biotin procedure with monoclonal antibodies (DAKO, Glostrup, Denmark). The relevant antibodies used for following antigens: CD5, CD20, CD23, CD10, CD79alpha,

BCL-2, BCL-6, MUM-1, and Ki-67. A negative staining was defined as the lack of color in target cells as it has been observed in a negative control sample. Negative controls were follicular centre cells of the reactive follicles. Cases with weak or focal staining were designated as positive. Expression of BCL-6 antigen was evaluated according to recommendations of WHO Classification (2008), with 30 % as a cut-off value for positivity. Proliferative cell activity was measured by expression of Ki-67 antigen in all tissue samples and graded numerically as percent of Ki-67 positive cells among total lymphoid cells. The statistical significance between analyzed parameters were tested by ANOVA multivariant test. The survival curves were estimated using the Kaplan–Meier method. The log rank test evaluated prognostic impact of analyzed parameters.

Results

In 41 investigated patients with relapsed DLBCL male/female ratio was 20/21. Mean age of patients was 53.0 ± 12.3 years with no significant difference according to gender. Relapsed patients with DLBCL were divided into two groups: the first who switched from BCL-6⁺ into BCL-6[−] (19/41) and the second BCL-6⁺ group or “non-switched” group (22/41) who stayed BCL-6⁺ in relapse and defined as GCB type of DLBCL (Fig. 1).

The presentation of relapsed DLBCL varied according to confirmatory biopsy as following: gastrointestinal tract (13 patients, 31.7 %), lymph node (9 patients, 22.0 %), liver and spleen (7 patients, 17.1 %) lungs (5 patients, 12.2 %), CNS (4 patients, 9.8 %), ovarian (2 patients, 4.8 %), vertebral (1 patient, 2.4 %). There were no differences in organ involvement between switched and non switched patients. Significantly higher percentage ($p < 0.05$) of bone marrow infiltration was recorded in BCL-6[−] patients (14/19, 73.7 %), compared to BCL-6⁺ patients (8/22, 36.3 %). During relapse of DLBCL, if transformed from BCL-6⁺ to BCL-6[−] pattern, biochemical findings typical for aggressive lymphomas were markedly increased, including significantly higher levels of LDH (1,832.5 ± 443.8 in BCL-6[−] vs. 457.3 ± 88.3 IU/L in BCL-6⁺ patients as control, $p < 0.01$) and CRP (35.9 ± 11.4 vs. 2.5 ± 0.7 mg/L, $p < 0.01$) (Fig. 2). Also, the average level of b2M was also higher in BCL-6[−] relapsed patients (8.4 ± 0.8 vs. 2.5 ± 0.8 mg/L, $p < 0.01$) (Fig. 2). Although we have found significantly higher uric acid average level in BCL-6[−] in comparison with BCL-6⁺ patients (287.2 ± 65.7 vs. 153.9 ± 33.1 µmol/L, $p < 0.01$), both values were still lower than upper limit of normal. Also, decreased levels of hemoglobin (91.7 ± 13.2 vs. 126.5 ± 8.6 g/L, $p < 0.01$), MCV (81.6 ± 20.2 vs. 92.0 ± 2.1 fL, $p < 0.01$) and hematocrit (0.29 ± 0.03 vs. 0.39 ± 0.03 %, $p < 0.05$) were found (Fig. 3). In BCL-6[−] vs. BCL-6⁺ results of other biochemical

Fig. 1 The immunohistochemical staining for BCL-6 expression in tumor tissue. **a** Positive; **b** negative

Fig. 2 Biochemical changes in relapsed DLBCL. **a** Levels of the LDH; **b** levels of the CRP and beta 2 microglobulin of BCL-6⁺ and switched into BCL-6⁻ patients in relapsed DLBCL

parameters were as following: ferritin ($2,735.6 \pm 628.2$ vs. 88.5 ± 20.2 $\mu\text{g/L}$, $p < 0.01$); Fe (11.2 ± 4.4 vs. 21.8 ± 7.6 $\mu\text{mol/L}$, $p < 0.01$); TIBC (35.5 ± 9.2 vs. 46.8 ± 7.0 $\mu\text{mol/L}$, $p < 0.01$) (Fig. 4).

Fig. 3 Markers of relapsed DLBCL. **a** Levels of the ferritin, **b** levels of the Fe and TIBC of BCL-6⁺ and switched into BCL-6⁻ patients in relapsed DLBCL

The distribution of IPI values in two groups of relapsed DLBCL were as follows: in BCL-6⁻ group IPI 2 (intermediate-low) had 2 patients, IPI 3 (intermediate-high) had

Fig. 4 Parameters of the anemia and tumor lysis syndrome. **a** Levels of the uric acid; **b** levels of the hemoglobin, MCV, and hematocrit (Ht) of BCL-6⁺ and switched into BCL-6⁻ patients in relapsed DLBCL

5 patients, IPI 4-5 (high) had 12 patients, while in BCL-6⁺ group IPI 2 had 9 patients, IPI 3 had 8 patients, IPI 4-5 (high) had 5 patients. These different distributions suggest that patients in BCL-6⁻ group are probably at a higher risk of poor outcome according to IPI.

The median Ki-67 expression, as antigen which reflects tumor proliferation, was 40 % (range 30–60 %) at the diagnosis of DLBCL, while the median Ki-67 expression at the time of relapse was 70 % (range 60–90 %) indicating higher proliferative capacity of neoplastic lymphoid cells in relapsed lymphoma comparing to initial values. In the first relapse the median Ki-67 expression in BCL-6⁻ patients was 75 % (range 60–90 %), while it was 70 % (range 60–90 %) in BCL-6⁺ patients, without significant differences between two groups ($p > 0.05$).

The average time to relapse (TTR) was 10.3 ± 4.6 months after initial diagnosis. It was shorter in the group of BCL-6⁻ (8.1 ± 4.2 months) versus BCL-6⁺ (14 ± 3.2 months) patients ($p < 0.05$). These findings were confirmed in survival analysis, indicating shorter TTR in BCL-6⁻ patients (log rank 46.181, $p = 0.000$; Fig. 5a). After second-line therapy

Fig. 5 Survival functions according to BCL-6 status in relapse. **a** Time to first relapse after the initial diagnosis (TTR1); **b** event free survival after the first relapse (EFSr); **c** overall survival since the first diagnosis (OS)

ESHAP, only 15.8 % (3/19) patients achieved CR in BCL-6⁻ group, while in BCL-6⁺ there was 31.8 % (7/22) patients ($p < 0.05$). Early second relapse, inside 12 months after achieving the second CR, occurred in all of the patients with BCL-6⁻ and only in two patients with BCL-6⁺. Survival analysis after the first relapse revealed that patients with BCL-6⁻ had significantly shorter event free survival in relapse (EFSr) (log rank 6.252; $p = 0.012$; Fig. 5b). Overall survival (OS) was also affected by BCL-6 switching in the first relapse. As a result, relapsed patients who remained BCL-6⁺ had significantly longer OS from the initial diagnosis (log rank 13.801; $p = 0.000$; Fig. 5c).

Discussion

To our best knowledge, in this study, for the first time we presented the occurrence of switching BCL-6⁺ protein expression to the BCL-6⁻ in DLBCL during disease relapse. All presented patients at the time of DLBCL diagnosis had BCL-6⁺ protein expression and have been treated with the R-CHOP chemotherapy. During relapse, 19 patients switched from BCL-6⁺ to BCL-6⁻. We concluded that switching from BCL-6⁺ to BCL-6⁻ could be immunohistochemical surrogate marker of the possible molecular triggering mechanisms during the course of very aggressive and treatment resistant disease. Thus, a transforming from BCL-6⁺ to BCL-6⁻ in relapsed DLBCL could be one of the molecular marker of disease aggressiveness and possible predictive marker for treatment options and consecutive outcome.

Terminal B-cell differentiation through the protein related domain M1 (PRDM1) repression was prevented by BCL-6 expression [11] as a hallmark of GC or GCB subtype of DLBCL and was associated with a favorable clinical outcome [1, 6]. There is some connection between BCL-6 rearrangement and non-GCB phenotype reported as ABC phenotype [12]. All of these conclusions are based on results of investigations that were performed on newly diagnosed DLBCL patients. In our study with relapsed DLBCL patients, we observed a switching from BCL-6⁺ into BCL-6⁻ in 19 patients, and this phenomenon was a predictor of poor outcome. As well, rearrangement of the BCL-6 may vary and depends on the chromosomal translocations [13].

A gene for BCL-6, also called LAZ3 or BCL6, is located on 3q27, and its rearrangement is frequently associated with aggressive course of DLBCL, but is also

found in a small proportion of low-grade B-NHL [14]. BCL-6 controls immune responses and promotes the formation of lymphomas, but its genomic targets for repression remain largely unknown [15]. Also, BCL-6 is a specific transcriptional repressor, and BCL-6-deficient mice have been reported to have Th2-type inflammatory diseases in multiple organs. The IL-18, a potent stimulator of Th2 cells is regulated by BCL-6 [16]. However, BCL-6 mutant mice cannot form GCs during a T cell dependent immune response and may have a fatal inflammatory disease with T helper type 2 cells [17, 18]. BCL-6 gene, a zinc-finger transcription factor can be translocated in DLBCL with both immunoglobulin (IGH, IGK, IGL) and non-Ig loci [7, 19]. However, the non-Ig/BCL-6 translocation results in replacement of its 5'-noncoding region with heterogeneous promoters, activating cell cycle progression. The non-Ig translocation has influence on the pattern of BCL-6 deregulation, thereby affecting the DLBCL clinical behavior. Thus, the non-Ig/BCL6 fusion is a poor prognostic indicator of DLBCL [20].

In this study, the most frequent localization in relapsed disease was gastrointestinal tract, the well known predilection place for aggressive B cell lymphomas [21]. In laboratory work-up, our BCL-6⁻ relapsed DLBCL patients had extremely elevated CRP and b2M, compared to BCL-6⁺. These biochemical parameters (CRP and b2M) are associated with poor prognosis of relapsed DLBCL. In literature data, significant portion of patients had increased value of b2M several months before the diagnosis of disease relapse [22]. In addition, these two markers are in correlation with clinical stage of NHL and presence of B symptoms [23].

Severe anemia could be the sign of bone marrow infiltration [24]. In this study, anemia was present in higher percentage of patients with BCL-6⁻ relapsed DLBCL and this correlated with significantly higher percentage of bone marrow infiltration in relapse.

The level of uric acid in the blood and its solubility depends on temperature and pH value [25]. Humans do not have uricase (urate oxidase), an enzyme which converts uric acid into soluble allantoin as a last end product of purine metabolism. Cancers represent robust system with very complex signaling networks specified for cell proliferation, migration, angiogenesis and apoptosis [26, 27]. When cancer cells are growing very fast and angiogenesis cannot follow growth of the cancer, tumor lysis occurs [26, 27], followed by significant increase of uric acid level. In our study uric acid was mainly higher in switched BCL-6⁻ relapsed patients, but the average level was still in referent range. Since the relapsed BCL-6⁻ patients had more aggressive course of the disease, it was also expected they will have higher level of Ki-67 expression as the indicator of tumor proliferation. However, in our study there was no

significant difference in median level of Ki-67 expression based on BCL-6 status and it was high both in BCL-6[−] and BCL-6⁺ group.

Modern diagnostic and treatment guidelines for DLBCL suggest that tumor biopsy should be obtained in patients who relapsed after more than 12 months since the initial diagnosis [28]. Moreover, cisplatin based regimens with rituximab followed by autologous stem cell transplantation are recommended treatment options in relapsed patients [28].

Results obtained in this study suggest that relapsed patients with anemia and hyperuricemia, elevated b2M, LDH and CRP, should be planned for biopsy even if they are in early relapse. In the case of the molecular switching (from BCL-6⁺ into BCL-6[−]), patients may be considered for aggressive non-platin based treatment (such as R-GE-MOX) [28]. However, the importance and significance of switched BCL-6 expression, still needs to be confirmed in larger and prospective clinical studies.

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