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Tissue microarray is inappropriate for analysis of BCL6 expression in diffuse large B-cell lymphoma

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ABSTRACT

Objective: In this study, our aim was to investigate how different immunohistochemical techniques may influence the result of BCL6 positivity and categorization in germinal center (GC) and non-GC derived diffuse large B-cell lymphoma (DLBCL), as it has been proposed that classification of DLBCL according to cell-of-origin by immunohistochemistry may be performed as a routine procedure in the diagnostic work-up. However, a number of technical issues need to be solved before introducing this as a standard technique.

Methods: Tumor specimens from 122 patients with de novo stage II-IV disease, adequately treated with anthracycline-containing chemotherapy regimens were collected. Immunohistochemical expression of BCL6, CD10 and MUM-1/IRF4 was examined using a tissue microarray (TMA) technique. BCL6 and CD10 were also evaluated on whole tissue sections.

Results: Due to profound tissue heterogeneity, BCL6 showed a wide range of positivity, with a high number of false negative results by TMA (25% positive), compared to 53% on whole tissue sections (WTS). CD10 was more homogeneously expressed, and TMA results corresponded better to WTS. Consequently, the results from categorization into GC and non-GC DLBCL differed considerably by use of the two methods, and resulted in very different outcome in terms of overall survival.

Conclusion: Immunohistochemical GC-status determined on TMA is not reliable enough to be used for individual treatment decisions in DLBCL, mostly due to difficulties in interpreting BCL6 status.

Key words: BCL6, diffuse large B-cell lymphoma, immunohistochemistry, tissue microarray

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma subtype. It is an aggressive lymphoma, heterogeneous with respect to morphology and clinical outcome. The current standard treatment is anthracycline-containing chemotherapy with the addition of rituximab.

Based on knowledge obtained from gene expression profiling, [1, 2] immunohistochemistry has been used to separate DLBCL into prognostically different subgroups based on cell of origin. The "germinal center (GC) phenotype" is characterized by CD10 and/or BCL6 expression[3], and associated with a better prognosis. The "activated B-like type" (ABC) is usually defined by absence of GC markers and presence of the MUM1/IRF4 antigen, a marker of plasmacytic differentiation, and is associated with an adverse clinical outcome.

A third group, "group 3", also associated with inferior survival, has been identified by gene expression profiling, genotypically located between the GC and ABC group[2]. The corresponding immunohistochemical features are not defined. For practical reasons it is usually combined with the ABC group, creating a "non-germinal center phenotype" (non-GC)[3].

The aim of the present study was to elucidate how different immunohistochemical techniques may influence the result of BCL6 and CD10 scoring and categorization in GC and non-GC derived DLBCL.

PATIENTS AND METHODS

Patients

Paraffin-embedded tumor tissue from 122 patients (64 males and 58 females) diagnosed with DLBCL, stage II-IV, during the period 1990-2002 at the University Hospitals of Lund, Uppsala and

Umeå were included in the analysis. Cases with primary CNS-involvement or primary mediastinal B-cell lymphoma or transformation from low-grade lymphoma were excluded. The DLBCL diagnosis was revised according to the WHO classification by a reference pathologist (ME). Patients were aged between 18 and 84 with a median age of 63 years.

Immunohistochemistry

Paraffin blocks were cut at 4-6 μm, dried over night at 60° and deparaffinised in xylene. Subsequently, sections were rehydrated through graded alcohol in water. Heat-epitope retrieval was achieved by boiling sections in EDTA buffer at pH 8.9 in a microwave oven at 800 W for 7 min and 300 W for 15 min. After boiling, sections were allowed to cool at room temperature for 20 min, rinsed thoroughly with water and placed in a Tris-buffered saline for 5 min. The primary antibodies were incubated for 25 min in room temperature. Endogenous peroxidase was blocked with peroxidase block solution provided in the EnVision kit for 25 min, and slides was rinsed with Tris-buffered saline. The immunostaining was performed using the Tech-Mate instrument (DAKO) and EnVision method (DAKO) according to the manufacturer´s instructions. For BCL6 staining with a conventional biotin-streptavidin method, LSAB (DAKO) was also performed [4].

BCL6 (DAKO, dilution 1:50), CD10 (NovoCastra, 1:100) and MUM1 (DAKO, 1:25) were analyzed on whole tissue sections (WTS) and in a tissue micro array system (TMA). For TMA, sections were stained with hematoxylin-eosin from each of tumor biopsy block, and areas of representative, non-necrotic sites were marked. From each tumor block, three 0.6 mm core biopsies were punched and positioned in a recipient paraffin block using a custom-made precision instrument. Stainings were interpreted as positive if >30% of tumor cells were positive. The fraction of BCL6-positive cells was estimated. The final conclusion of positive or negative staining was based on an estimated mean of the whole section. Positive controls were run in parallel with the staining of the sections to rule out false negative results.

Classification into GC and non-GC DLBCL was performed using the algorithm proposed by Hans et al [3].

RESULTS

The results of immunohistochemistry are summarized in Table 1.

By use of a conventional biotin-avidin method (LSAB), BCL6 was positive in 12% on TMA sections. Using a more sensitive technique, EnVision, and the same TMA-blocks, BCL6-positivity was twice as high, 25%.

On WTS, stained for BCL6, it was apparent that a high number of positively stained nuclei were concentrated in focal areas, up to 70-80% in selected fields of view, while other areas displayed less than 10% positive nuclei (Figure 1). Consequently, on WTS, BCL6 status changed from negative to positive in 29% and was considered positive in 53% of the cases.

By TMA and EnVision, CD10 was positive in 37 % of the cases. On WTS, CD10 status changed from positive to negative in six percent and from negative to positive in two percent and was considered positive in 33%. For MUM1, there was no difference between TMA and WTS, due to homogenous staining.

By the use of WTS, 47% were classified as GC-DLBCL, which were associated with a superior OS. Median survival for non-GC and GC cases was 32.5 and 121 months respectively (p=0.006).

By TMA, 40 % were classified as GC-DLBCL. Median survival for non-GC and GC cases according to TMA results was 44 and 62 months respectively (p=0.19), i e not significantly different.

DISCUSSION

The algorithm for creating an immunohistochemical GC-profile proposed by Hans et al[3], uses the combination of the three markers CD10, BCL6 and MUM1/IRF4. Several studies[5, 6] , but not all [7-9] have found significant survival benefits for patients with a GC-profile, in some series using other cut-off values, or using CD138 as an additional marker of non-GC origin. These studies have been performed in patients treated with chemotherapy alone. The current standard of care for DLBCL is chemoimmunotherapy, including the CD20-antibody rituximab, and one may argue that the prognostic impact of GC-profiling in the rituximab era is not yet settled.

However, from a tumor biological standpoint, DLBCL should be regarded as at least two biologically separate entities, possibly benefiting from different treatment approaches.

Hence, it may be suggested that immunohistochemical profiling should be a part of the diagnostic assessment of DLBCL, in clinical routine and in clinical trials.

However, our present results show that there are several problems to be solved before this method is introduced into clinical practice.

Firstly, BCL6-positivity depends on the immunohistochemical technique used. We found that the use of the EnVision method (DAKO) renders a much higher degree of positivity than using the same antibody in a traditional biotin-streptavidin method (LSAB).

Secondly, and more importantly, we found a great discrepancy between BCL6 status determined on tissue microarrays and whole tissue sections. Examined on whole sections, BCL6 status changed in 29% of the cases. One explanation could result from poor TMA-sampling, resulting in false negative results. However, the core biopsies were initially scrutinized and not accepted for inclusion if the material was unrepresentative. Another, more probable explanation lies in the fact that BCL6 displayed a very heterogeneous staining pattern. Within the same tumor, we found hot spots

containing 70-80% positively stained nuclei, while in other areas less than 10% of the nuclei were positive. This makes the TMA technique inappropriate for immunohistochemical evaluation of BCL6 expression, due to a high risk for non-representative core biopsies, although it is possible that a higher number of core biopsies may reduce the number of false BCL6-negatives.
Possibly due to these difficulties, the international Lunenburg Lymphoma Biomarker Consortium found a very low reproducibility for BCL6 expression in DLBCL, compared to other markers[10]. When CD10 was analyzed both on TMA and WTS, there was a lack of concordance in 9% of the cases, a somewhat more reasonable discrepancy, within the expected margins of error [11, 12].

In conclusion, due to the difficulties described, immunohistochemical GC-status determined on tissue microarrays is not reliable enough to be used for individual treatment decisions in DLBCL.

REFERENCES

1. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J, Jr., Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling: Nature. 2000; **403**: 503-511.

2. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Giltnane JM, Hurt EM, Zhao H, Averett L, Yang L, Wilson WH, Jaffe ES, Simon R, Klausner RD, Powell J, Duffey PL, Longo DL, Greiner TC, Weisenburger DD, Sanger WG, Dave BJ, Lynch JC, Vose J, Armitage JO, Montserrat E, Lopez-Guillermo A, Grogan TM, Miller TP, LeBlanc M, Ott G, Kvaloy S, Delabie J, Holte H, Krajci P, Stokke T, Staudt LM. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 2002;**346**:1937-1947.

3. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, Pan Z, Farinha P, Smith LM, Falini B, Banham AH, Rosenwald A, Staudt LM, Connors JM, Armitage JO, Chan WC. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 2004;**103**:275-282.

4. Boenisch T. Immunochemical Staining Methods, ed 3rd, 2001.

5. Barrans SL, Carter I, Owen RG, Davies FE, Patmore RD, Haynes AP, Morgan GJ, Jack AS. 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.

6. Chang CC, McClintock S, Cleveland RP, Trzpuc T, Vesole DH, Logan B, Kajdacsy-Balla A, Perkins SL. 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.

7. Colomo L, Lopez-Guillermo A, Perales M, Rives S, Martinez A, Bosch F, Colomer D, Falini B, Montserrat E, Campo E. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. Blood 2003;**101**:78-84.

8. Linderoth J, Jerkeman M, Cavallin-Ståhl E, Kvaloy S, Torlakovic E. Immunohistochemical expression of CD23 and CD40 may identify prognostically favorable subgroups of diffuse large B-cell lymphoma: a Nordic Lymphoma Group Study. Clin Cancer Res 2003; **9**:722-728.

9. Hirose Y, Masaki Y, Karasawa H, Shimoyama K, Fukushima T, Kawabata H, Ogawa N, Wano Y, Ozaki M. Incidence of diffuse large B-cell lymphoma of germinal center B-cell origin in whole diffuse large B-cell lymphoma: tissue fluorescence in situ hybridization using t(14;18) compared with immunohistochemistry. Int J Hematol 2005;**81**:48-57.

10. de Jong D, Rosenwald A, Chhanabhai M, Gaulard P, Klapper W, Lee A, Sander B, Thorns C, Campo E, Molina T, Norton A, Hagenbeek A, Horning S, Lister A, Raemaekers J, Gascoyne RD, Salles G, Weller E. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications--a study from the Lunenburg Lymphoma Biomarker Consortium. J Clin Oncol 2007;**25**:805-812.

11. Hedvat CV, Hegde A, Chaganti RS, Chen B, Qin J, Filippa DA, Nimer SD, Teruya-Feldstein J. Application of tissue microarray technology to the study of non-Hodgkin's and Hodgkin's lymphoma. Hum Pathol 2002;**33**:968-974.

12. Zettl A, Meister S, Katzenberger T, Kalla J, Ott MM, Muller-Hermelink HK, Ott G. Immunohistochemical analysis of B-cell lymphoma using tissue microarrays identifies particular phenotypic profiles of B-cell lymphomas. Histopathology 2003;**43**:209-219.

Table 1

Results of immunohistochemical stainings for BCL6 and CD10 on tissue microarray (TMA) and whole tissue sections (WTS) and with different staining methods

Epitope	Staining method	n	% positive >30% cut-off
BCL6 (TMA)	EnVision	122	25
BCL6 (WTS)	EnVision	122	53
BCL6 (TMA)	LSAB	122	12
CD10 (TMA)	EnVision	122	37
CD10 (WTS)	EnVision	122	33
MUM1 (TMA)	EnVision	122	43

FIGURE LEGENDS

Figure 1

This shows two different areas (40X magnification) of a whole tissue section of a 40 tumor with heterogeneous BCL6 expression. In panel (A) more than 80% of the lymphoma cells express BCL6. The area showed in panel (B) is almost completely BCL6-negative.

Figure 1