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

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Background and Objectives. Patients with diffuse large B-cell lymphoma (DLBCL) could benefit from integration of well-established bioclinical prognostic factors with new tools – such as micro-arrays – exploring aberrant gene and/or protein expression.

Design and Methods. Tissue micro-arrays (TMA) were constructed for the paraffin blocks of 68 patients with *de novo* DLBCL with nodal presentation, who underwent MACOP-B, and for whom complete clinical information was available. TMA were tested with specific antibodies against CD10, CD20, CD30, CD79a, CD138, Bcl-2, Bcl-6, IRF4, and IRTA1.

Results. The following phenotypic subclassification was made: a) CD10$^+$/Bcl-6$^+$ or Bcl-6$^+$/IRF4$^+$, but Bcl-2$^-$/CD30$^-$/CD138$^-$ suggesting B cells gathering/leaving the germinal center (group 1; n=36); b) Bcl-2$^+$/CD10$^-$/Bcl-6$^-$ and CD30$^+$ or CD138$^+$ corresponding to putative non-germinal center B cells with features of activation or plasmablastic/plasmacellular differentiation (group 2; n=17); c) CD30–/CD138– with extensive Bcl-2 positivity and variable CD10, Bcl-6 and IRF4 combinations (group 3; n=15). Mean IPI scores were 0.6, 1.9 and 1.1 for groups 1, 2 and 3, respectively (*p*=0.001). Complete remission (CR) rates were 89%, 53% and 73% (*p*=0.015). The 3-year relapse-free survival (RFS) rates are 86%, 41% and 63% (*p*=0.001) and 42-month overall survival (OS) rates are 91%, 38% and 66% (*p*=0.0002).

Interpretation and Conclusions. The present TMA-based study suggests an immunophenotypic profiling system for patients with *de novo* DLBCL that seems to provide additional prognostic information and contributes to the existing debate on the identification of suitable immunohistochemical surrogates of gene expression profiling results.

Key words: TMA, DLBCL, predictive factors.

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Both the classification and treatment of diffuse large B-cell lymphoma (DLBCL) are the object of continuous refinements based on the ongoing advances in our biological and clinical knowledge of the condition. However, the development of more accurate prognostic indicators – such as the International Prognostic Index (IPI)[1] – allowing the calibration of treatment to the real needs of the single patient has yet to be translated into a higher probability of cure. Currently, both clinicians and pathologists hope to substantially improve patients' outcomes by integrating the traditional biological and clinical prognostic factors with the highly attractive new gene expression profiling techniques.[2-9] In 2000, Alizadeh *et al*.[2] first reported on the application of cDNA micro-array technology to DLBCL by assessing the levels of expression of thousands of genes in mRNA extracted from 44 frozen samples. In particular, they proposed that patients with a germinal center B-cell-like (GCB) signature might have a better outcome than those with an activated B-cell-like profile (ABC).[2] In 2002, use of the oligonucleotide microarray strategy led Shipp *et al*.[3] to suggest that the outcome of 77 DLBCL treated with CHOP-like chemotherapy regimens had been influenced by gene expression profiles other than the histogenetic ones. Later on, based on the analysis of 274 cases Rosenwald *et al*. established that the overall survival in DLBCL was more accurately predicted by gene expression signatures that reflected not only the cell of origin, but also proliferation rate and host immune response to the tumor.[4] The same group recognized another cluster of DLBCL (termed *type 3* or *unclassified*),

which did not fit with the GCB and ABC ones, and found that t(14;18) and amplification of the *c-rel* locus were associated with the GCB profile, thus suggesting that the three identified clusters corresponded to pathogenetically distinct diseases.[4-6] The concept that DLBCL is indeed a multifaceted entity has further been strengthened by the detection of specific profiles for the primary mediastinal and AIDS-related varieties.[7-9] Even though gene expression profiling (GEP) techniques have dramatically improved our knowledge of lymphoid tumor biology and outcome, they still suffer from practical limitations, such as their high costs and the need for fresh or optimally cryopreserved samples, which currently preclude widespread routine application.[10] Aiming to provide a more accessible approach, Lossos *et al.* have focused their attention on six genes (*LMO2, BCL6, FN1, CCDN2, SCYA2,* and *BCL2*) and found that measurement of their expression might be sufficient to predict overall survival in DLBCL.[11]

Tissue micro-array (TMA) technology (as developed at the Institute of Pathology in Basel) allows simultaneous, high-throughput immunohistochemical analysis of protein expression in a large number of specimens on a single slide.[12] So far, this technology has been successfully used for the construction of TMA comprising several hundreds of cases of Hodgkin's disease or non-Hodgkin's lymphoma and shows the same consistency as conventional sections.[13-20] Thus, TMA could provide an ideal tool for the validation of GEP studies on a large scale basis and the search for protein surrogates of the deregulated genes, which can be readily and inexpensively identified on routine (formalin-fixed, paraffin-embedded) tissue samples under standardized conditions.[10,17-20]

We constructed a TMA from 68 homogeneously treated cases of *de novo* DLBCL with nodal presentation, aiming to assess whether immunophenotypic profiles might be predictive of the clinical outcome.

Design and Methods

Patients

All patients with a diagnosis of *de novo* DLBCL[21] with nodal presentation, who had been treated at the Institute of Haematology and Clinical Oncology "L. and A. Seràgnoli" of Bologna University between 1999 and 2002, were screened for the study. Those patients with complete clinical information and sufficient formalin-fixed, paraffin-embedded pathologic material[22] for TMA analysis were considered evaluable. Accordingly, 68 immunocompetent patients (36 males and 32 females; median age 48 years, range 19–62) were evaluated. Twenty had stage II disease and 48 had stages III–IV. Only 10 (14%) had bone marrow involvement. The IPI score was 0 in 15 cases, 1 in 15 cases, 2 in 17 cases, 3 in 14 cases, 4 in 5 cases, and 5 in the remaining 2 cases. The performance status (PS) was 1 in all patients. The median follow-up was 39 months (range: 24–54). All patients received the MACOP-B regimen[24] as front-line treatment, in keeping with our institutional protocol, and completed the scheduled therapy that did not include anti-CD20 antibodies.

TMA construction

For TMA construction, a hematoxylin and eosin (H&E)-stained slide was cut from each paraffin-block and reviewed by at least two experienced hematopathologists. Representative tumor regions were morphologically identified and marked on the H&E stained slides. Tissue cylinders with a diameter of 0.6 mm were punched from the marked areas of each block and brought into a recipient paraffin block, using a precision instrument (Beecher Instruments, Silver Spring, MD, USA) as previously described.[13-17] To overcome the problem of tissue micro-heterogeneity and increase the number of evaluable cases, each donor tissue block was punched six times for the construction of three recipient blocks, which contained 136 tissue cores each. Sections 4 μm-thick were cut from each TMA and transferred to electrically-charged slides. One section/TMA was stained with Giemsa (Figure 1A).

Immunohistochemistry

Following appropriate antigen retrieval,[24] the sections obtained from each TMA were tested with specific antibodies against CD10 [clone 56C6; Novocastra, (Newcastle, UK); dilution 1:5], CD20 [clone L26; DakoCytomation (Denmark); dilution 1:200], CD30 [clone Ber-H2; kindly provided by Prof. H. Stein; dilution 1:2], CD79a [clone JCB117; kindly provided by Prof. D.Y. Mason; dilution 1:8], CD138 [clone 5F7; Novocastra (Newcastle, UK); dilution 1:20], Bcl-2 [clone 124; kindly provided by Prof. D.Y. Mason; dilution 1:4], Bcl-6 [clone PG-B6p; kindly provided by Prof. B. Falini; dilution: 1:2], IRF4/MUM1 [clone MUM-1p; kindly provided by Prof. B. Falini; dilution 1:2], and IRTA1 [clone M-IRTA1, kindly provided by Prof. B. Falini; dilution 1:2]. Bound antibodies were visualized by the alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes technique.[25] For positive controls, sections from reactive lymph nodes and tonsils were tested in parallel. For negative controls, the primary antibodies were omitted.

Criteria for marker evaluation

Each section was independently evaluated by at least two experienced hematopathologists. According to the criteria used by Hans *et al.*,[20] cases were considered positive if 30% or more of the tumor cells
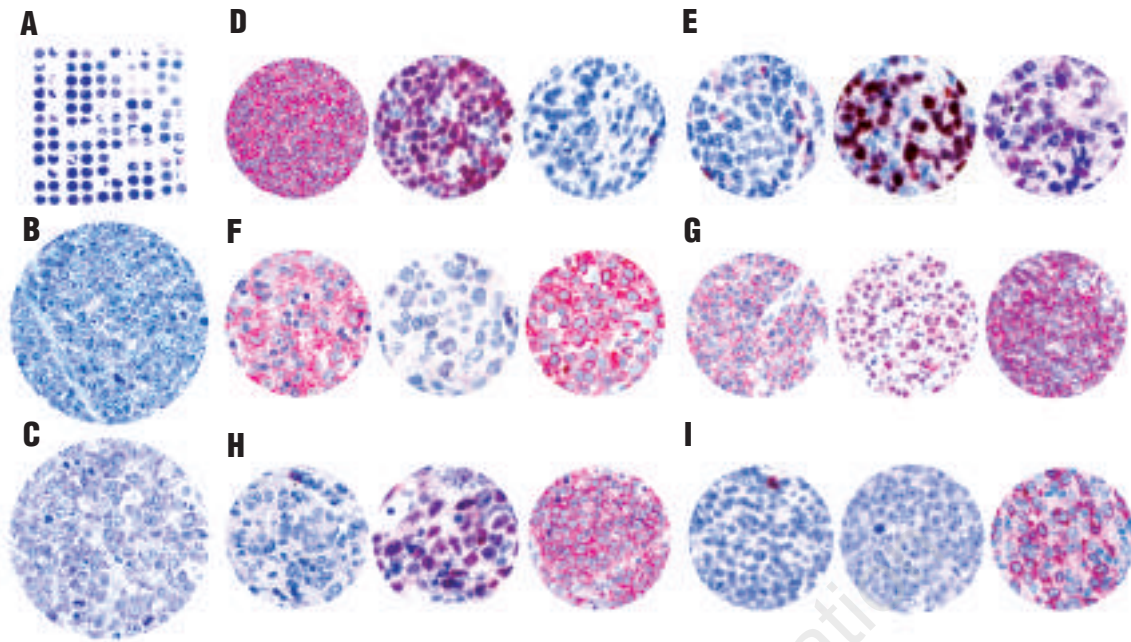
Figure 1. A: partial panoramic view of one of the tissue micro-arrays (Giemsa, x2); B: CD10, Bcl-6, and Bcl-2 immunostains in a DLBCL belonging to group 1 and showing a germinal center B-cell (GCB) phenotypic profile; C: CD10, Bcl-6 and IRF4 immunostains in a case also included in Group 1; C: CD30, Bcl-2 and CD10 immunostains in a DLBCL entering group 2; D: CD138, Bcl-2 and IRF4 positivities in another case belonging to the same group; E: CD10, Bcl-6 and Bcl-2 positivities in a case enrolled in group 3; F: CD10, Bcl-6 and Bcl-2 immunostains in another case entering the same group (APAAP technique; Gill's hematoxylin counterstain).

were stained with an antibody. The number of positive cells was estimated by each observer. The insensity of staining was also evaluated, but was not used to determine positivity, as it can vary depending on tissue fixation and putative normal counterpart of the tumor. Discordant results among observers were registered and made the object of collective discussion at a multi-head microscope; this involved counting the number of positive cells out of the total number of cells comprised within each tissue core. The TMA results obtained with the antibodies against CD20 and CD79a were compared with the recorded results of conventional immunostained preparations at the time of diagnosis.

Statistical analysis

Complete response (CR) was defined according to International Working Group Recommendations.[26] Overall survival (OS) was measured from entry into the protocol until the last follow-up or death. Relapse-free survival (RFS) was measured from the date of CR until relapse or the last follow-up or death. OS and RFS curves were calculated according to the Kaplan and Meier method.[27] The significance of the differences between the curves was estimated by the log-rank test.[28] The χ^2 test was used according to Mantel and Haenszel.[29] Two-sided p values were used throughout.

Results

TMA reliability and accuracy

All 68 cases were representative by Giemsa morphology. There was no tissue on 32 positions. However, since each donor block had been punched six times, at least one evaluable core was present in all instances. The comparison between the immunostains performed on TMA and conventional sections showed 100% concordance as regards the B-cell markers, CD20 and CD79a. Inter-observer discrepancies in the estimates of positive cells never exceeded 5% and were limited to a few cores: consensus was always easily reached during the collective discussion. Notably, the percentage of cells stained for each marker more often exceeded the 30% cut-off value (as shown in Figure 1), thus allowing easy classification of the case.

Based on the reported different expression of the investigated molecules in normal and neoplastic lymphoid tissue,[13,19,20,30-40] the 68 cases were sub-classified into three groups. In detail, group 1 included 36 cases that were either CD10+/Bcl-6+ (11 cases) or Bcl-6+/IRF4+ (25 cases), but lacked Bcl-2, CD30 and CD138 (Figures 1B and 1C). Interestingly, two of the CD10+/Bcl-6+ tumors did also express IRF4. The profile of this group was regarded as consistent with B-cells entering into the formation of or on the way to

Table 1. Complete response (CR), relapse rate, 3-year OS, and 3-year RFS of the 3 phenotypic subgroups.

Group	N.	CR (%)	Relapse (%)	3-yr OS	3-yr RFS
1	36	32/36 (89 %)	3/32 (9 %)	91%	86 %
2	17	9/17 (53%)	5/9 (55 %)	38%	41 %
3	15	11/15 (73 %)	4/11 (36 %)	66%	63 %

Figure 2. Overall survival curves: comparison among TMA groups 1, 2 and 3.

Figure 3. Relapse-free survival curves: comparison among TMA groups 1, 2 and 3.

leaving the germinal center. Group 2 comprised 17 cases with expression of either CD30 (12 cases) or CD138 (5 cases), all of which were Bcl-2$^+$ and CD10$^-$/Bcl-6$^-$ (Figures 1D and 1E); it should be noted that CD138$^+$ tumors also expressed IRF4, whereas the CD30$^+$ ones did not. Among the latter tumors, there were 3 cases completely consisting of CD30$^+$ cells and 9 cases that contained more than 30% CD30$^+$ elements. The latter simultaneously expressed the IRTA1 molecule[41] in the majority of neoplastic cells (*data not shown*). The lymphomas in this group were thought to consist of non-germinal center B-cells showing features of activation (CD30$^+$) or plasmablastic/plasmacellular differentiation (CD138/IRF4$^+$). Finally, group 3 contained 15 cases that were regularly Bcl-2$^+$, CD30$^-$ and CD138$^-$, but displayed variable combinations of the remaining markers: CD10$^+$/Bcl-6$^+$/IRF4$^-$ (3 cases), CD10$^-$/Bcl-6$^+$/IRF4$^+$ (6 cases), CD10$^-$/Bcl-6$^+$/IRF4$^-$ (4 cases) or CD10$^-$/Bcl-6$^-$/IRF4$^+$ (2 cases) (Figures 1F and 1G). This group included cases of putative germinal center or post-germinal center B-cell derivation, all gathered by Bcl-2 positivity of most if not all neoplastic elements, a characteristic that has previously been proposed as a poor prognostic indicator (*for comprehensive reviews see refs. no. 13 and 36*).

Clinico-phenotypic correlations

The three groups all showed significant differences in terms of clinical behavior and response to therapy. The mean IPI values of the patients belonging to group 1, 2 and 3 were 0.6, 1.9 and 1.1, respectively (*p*=0.001). Table 1 summarizes the CR rates, the relapse rates, and the 3-year OS and RFS data for each group. The global CR rate was 76% (52/68); at a median follow-up of 39 months (range 19-52), 40/68 (59%) patients are still in continuous first CR, with a relapse rate of 23% (12/52). On immunophenotypic grounds, 32/36 (89%) patients in group 1 achieved CR and, at a median follow-up of 38 months (range 20-52), only 3/32 (9%) have relapsed. In group 2, 9/17 (53%) patients achieved CR, but 5/9 (56%) have relapsed (median follow up 40 months, range 19-50). In group 3, 11/15 (73%) patients
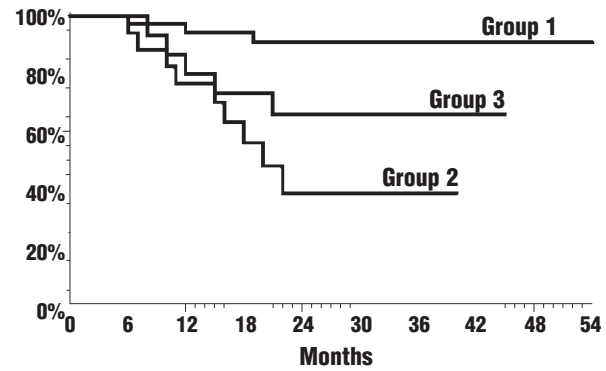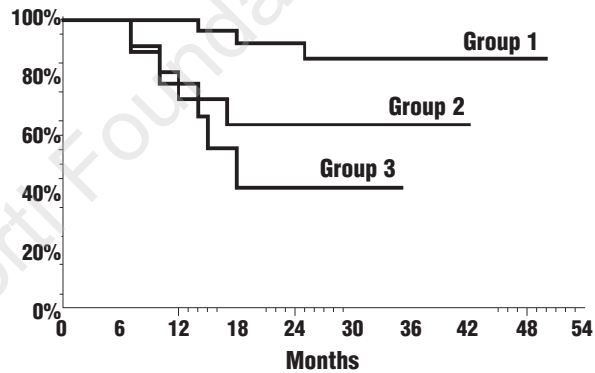
obtained CR after front-line chemotherapy and 4/11 (36%) have relapsed (median follow up 39 months, range 21-49). The difference among the CR rates (group 1: 89%; group 2: 53%; group 3: 73%) is statistically significant (*p*=0.015), as is that among the relapse rates (group 1: 9%; group 2: 55%; group 3: 36%) (*p*=0.007). Figure 2 shows the different OS curves calculated with respect to the TMA subsets. At 42 months, the OS rates are 91% for group 1, 38% for group 2 and 66% for group 3 (*p*= 0.0002). Figure 3 depicts the curves for 3-year EFS (group 1: 86%; group 2: 41%; group 3: 63%; *p*=0.001).

Discussion

The IPI was originally developed in a series of 3,273 patients with different kinds of aggressive lym-

phoma.[1] Based on the analysis of a broad array of clinical/laboratory parameters, including sex, age, lactate dehydrogenase, performance status, number of extranodal sites, albumin, β2 microglobulin, bulky disease, stage, and B symptoms, a restricted set of independently relevant parameters was selected to constitute a prognostic index. Limitations of the IPI regard the characteristics of the original data set and the current availability of new parameters. In fact, since 1987 more sensitive diagnostic procedures have come into use, treatment has been improved, and evaluation of response has changed. Moreover, new treatment strategies could require tailored prognostic profiles with other dominant parameters.[42] Therefore, the time seems to have come to start to move the IPI into a more biologically based index. Cancer patients within the same diagnostic category often show significant differences in response to therapy, and this clinical heterogeneity can be due to molecular/biopathologic differences among their tumors. In DLBCL (the most common type of non-Hodgkin's lymphoma in adults), initial GEP studies revealed that this single diagnostic category actually contains distinct diseases that differ in the expression of hundreds of genes. Rosenwald and Staudt pioneered gene expression profiling in DLBCL by showing that there are three possible biological entities: the GCB type expressing genes restricted to the germinal center, the ABC type expressing genes typical of B cells undergoing activation and/or plasma cell differentiation, and a third type with intermediate characteristics between the GCB and ABC ones.[4-6]

The TMA technique[10,13-20] represents an ideal integration of GEP techniques. Two independent TMA-based studies on DLBCL[19,20] which identified protein surrogates of deregulated genes have recently been published. The former of these studies proposed 8 biological markers (cyclin E, CDK1, SKP2, EBER, MUM1, CDK2, Bcl-6, and Rb-P) that can improve the capacity for predicting failure and survival in combination with IPI. Notably, the results of Sáez et al. «are difficult to match with the three DLBCL subgroups defined by Rosenwald et al.»[19] The other study was based on a TMA containing 152 DLBCL, 142 of which had been successfully evaluated by cDNA microarray.[20] Cases were subclassified using CD10, Bcl-6, and MUM1 expression, and 64 cases were considered GCB and 88 cases non-GCB. The 5-year OS for the GCB group was 76% whereas that of the non-GCB one was only 34%. Bcl-2 and cyclin D2 were adverse predictors in the non-GCB group.[20]

Three further studies have been carried out on conventional tissue sections – rather than TMA – always with the scope of identifying immunohistochemical GEP surrogates.[30-32] Colomo et al. analyzed 128 DLBCL by applying an antibody panel similar to that of the present report.[30] The percentages of CD10, Bcl-6 and IRF4 positivities actually corresponded to those observed by our group (21% vs. 20%, 72% vs. 70%, and 54% vs. 56%), as did the main phenotypic profiles. Only the incidence of CD138 expression turned out to be higher in our series (7% vs. 2%). In line with our observations, Bcl-2 positivity was significantly associated with higher Ann Arbor and IPI stages and shorter overall survival.[30] In contrast, the remaining markers lacked prognostic relevance.[30] Such a discrepancy might reflect the different case mix: in fact, Colomo et al. analyzed a rather heterogeneous series that included 50 extranodal tumors and patients who had been treated with different therapeutic combinations (with and without adriamycin).[30] By examining 125 DLBCL, Linderoth et al. also found an association of Bcl-2 expression with more aggressive disease and lack of prognostic relevance of the CD10, Bcl-6 and CD138 molecules.[31] However, their study had some possible pitfalls: firstly, it included nodal and extranodal tumors (without reporting the corresponding rates) and secondly a low cut-off value of 10% was used to define positivity.[31] This latter decision may be critical for markers such as Bcl-6, which is also expressed by a proportion of T-lymphocyte:[38] by applying a more selective value (30% in Hans' series and in our own and 25% in Colomo's) the percentage of Bcl-6-positive DLBCL would likely be lower than 97%. Finally, Chang et al. analyzed 42 DLBCL by applying antibodies against CD10, Bcl-6, IRF4, and CD138.[32] Based on the marker combination, the cases were subdivided into three groups showing expression of: (i) CD10 and/or Bcl-6 alone; (ii) CD10 or Bcl-6 plus IRF4 or CD138, and (iii) IRF4 and/or CD138 in the absence of CD10 and Bcl-6.[32] The first group had a significantly better clinical course than did the remaining two.[32]

In the present study, 68 cases of de novo DLBCL with nodal presentation, which had been homogeneously treated with the MACOP-B regimen and followed for at least two years, were categorized into three prognostically different subsets on the basis of Bcl-2/Bcl-6/CD10/IRF4/CD30/CD138 expression patterns observed on TMA. The immunohistochemical profile of the subset that showed the best clinical course (89% CR, 86% 3-year RFS and 91% 42-month OS) was consistent with B cells gathering/leaving the germinal center (i.e. CD10+/Bcl-6+ or Bcl-6+/IRF4+), but lacking Bcl-2, CD30 and CD138. The poorest clinical course (53% CR, 41% 3-year RFS and 38% 42-month OS) was found among those patients who showed an immunohistochemical profile suggestive of non-germinal center B cells with features of activation or plasmablastic/plasmacellular differentiation (either CD30 or CD138 positivity accompanied by Bcl-2 expression and CD10 and Bcl-

6 negativity). A third immunohistochemical profile, mainly characterized by extensive Bcl-2 positivity associated with CD30 and CD138 negativity, showed an intermediate clinical course (73% CR, 63% 3-year RFS and 66% 42-month OS).

Interestingly, our analysis was based on the same molecules as those included in Hans' display,[20] integrated by CD30 and CD138. CD30 is a member of the tumor-necrosis factor-receptor superfamily, long since recognized as an activation marker of both B- and T-lymphocytes.[33,34] Notably, an interfollicular large B-cell component with dendritic morphology has recently been described in the lymph node; it expresses CD30 at variable levels and lacks both germinal center and plasma cell markers. It has been proposed as the normal counterpart of some large B-cell tumors consisting of activated lymphoid elements, including common Hodgkin's disease.[43] Furthermore, CD30 can be detected in a proportion of nodal marginal-zone lymphomas, especially of large-cell size (*Marafioti T, Mason DY, Falini B and Pileri SA, manuscript in preparation*). Remarkably, most of our CD30-positive DLBCL did also express the IRTA1 molecule (*data not shown*), which has recently been described in marginal zone B cells of mucosa-associated lymphoid tissue and monocytoid B lymphocytes of the lymph node.[41] CD138 is a well-known marker of plasmablastic/plasmacellular differentiation,[13,35-37] also included in Chang's display.[32]

Unlikely Colomo et al.[30] and Hans et al.,[20] we did not regard IRF4 as a post-follicular marker: in fact, its expression physiologically starts at the edge of the germinal center to reach its peak during plasma cell differentiation.[39] In keeping with this assumption, we considered the co-expression of Bcl-6 and IRF4 as consistent with a cell in transit through the germinal center. The same viewpoint was taken by Chang et al. in their study.[32] Secondly, in line with previous immunohistochemical reports (*for comprehensive reviews see refs. no. 13 and 36*) and the recent inclusion of *BCL-2* among the genes whose expression significantly affects the outcome of DLBCL,[11] we paid special attention to the possible negative influence of Bcl-2 protein on response to therapy and survival.

Based on these principles, we grouped our cases in a partly different way to that followed by Hans et al.[20] In particular, we identified 3 groups of patients with highly significant differences in terms of CR, RFS and OS rates, whose clinical course roughly parallels one of the 3 subsets (GCB, ABC and unclassified) defined by gene expression profiling studies.[4-6] Notably, the segregation of Bcl-2⁻ from Bcl-2⁺ tumors among our putative germinal center B-cell-derived cases allowed us to identify a group of patients with a very favorable clinical course and excellent response to therapy (OS rate: 91%). In this respect, our approach seems to be even more effective than the one of Hans et al.[20] without being in contrast with it: in fact, by gathering our Bcl-2⁺ and Bcl-2⁻ germinal center B-cell-derived tumors we would obtain the same OS rate as the one shown by these authors for their GCB group (76%) (*data not shown*). Within this frame, one can also speculate that the lack of Bcl-2 protein evaluation might explain the different behaviors observed by Chang et al. among their *activated GC B-cell tumors* (characterized by co-expression of germinal center B-cell markers and IRF4).[32] In fact, in spite of the common phenotype 5/11 patients achieved stable CR, while the remaining ones died of their disease.[32]

In conclusion, our study further confirms the usefulness and reliability of the TMA technique in the straightforward analysis of large series of lymphoid tumors. In addition, as endorsed by Hans et al.,[20] it contributes to the existing debate on the identification of suitable immunohistochemical surrogates of gene expression profiling results, capable of identifying subsets of patients with different biological risks and different therapy requirements.

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