ORIGINAL ARTICLE

Prognostic significance of CD44 expression in diffuse large B cell lymphoma of activated and germinal centre B cell-like types: a tissue microarray analysis of 90 cases

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Background: Gene expression profiling of diffuse large B cell lymphoma (DLBCL) revealed three disease types: germinal centre B cell-like (GC), activated B cell-like (ABC), and a "third" type. Expression of CD44 variant isoforms (CD44v) is associated with an unfavourable clinical outcome in DLBCL, but previous studies did not consider the clinicopathological heterogeneity of this disease.
Aims: To analyse the expression and prognostic significance of CD44 in DLBCL types.
Methods: A tissue microarray (TMA) comprising 90 DLBCLs was constructed. CD10, CD20, bcl-2, bcl-6, CD44 standard isoform (CD44s), and CD44v4, CD44v6, and CD44v9 were analysed immunohistochemically and correlated with clinical follow up.
Results: TMA expression of CD10, CD20, bcl-2, and bcl-6 showed 100% concordance with results from conventional sections in 60 cases. Samples were segregated into 22 GC (bcl-6+/CD10+/bcl-2–), 25 ABC (bcl-6–/CD10–/bcl-2+), and 35 unclassifiable DLBCLs. Overall survival (OS) at 30 months was 89%, 44%, and 58% in GC, ABC, and unclassified types, respectively. CD44v6 was coexpressed with bcl-2, appeared predominantly on bcl-6 negative cases, and correlated with disease stage. Cases negative for CD44s could be separated into CD44v6 negative (OS, 82% at 70 months) and CD44v6 positive (OS, 58%).
Conclusions: TMA technology is useful for immunophenotyping and clinicopathological analysis of large lymphoma populations. The GC phenotype of DLBCL is of independent prognostic significance for OS. Expression of CD44v6 correlates with disease stage, and might contribute to lymphoma dissemination. CD44v6 is expressed predominantly in ABC DLBCL, and in CD44 negative cases is associated with worse OS.

Diffuse large B cell lymphoma (DLBCL) is the most common lymphoid malignancy, comprising 35–40% of adult non-Hodgkin lymphomas (NHLs).[1] Although listed as a specific entity in the World Health Organisation classification,[1] it represents a heterogeneous disease—morphologically, phenotypically, genetically, and clinically. DLBCL either develops de novo, or occurs as a consequence of low malignant B cell diseases.[1 2] Recent gene expression profiling of DLBCL using cDNA and oligonucleotide microarrays revealed that this single entity can be divided into three categories: DLBCL of germinal centre B cell-like type (GC), DLBCL of activated B cell-like type (ABC), or a "third" type.[3–6] Among the differentially expressed genes within the ABC and GC subgroups, CD44 is of particular interest.[6] CD44 standard isoform (CD44s) and its variants (CD44v) represent cell surface glycoproteins, which are generated by the alternative splicing of CD44 mRNA. These molecules play a key role in lymphocyte migration, homing, and activation and participate in the transmission of signals that regulate haemopoiesis and apoptosis. They are either constitutively expressed on the surface of peripheral lymphocytes (CD44s) or are induced upon antigen mediated activation (CD44v3 and CD44v6) (reviewed by Sneath and Mangham[7]). CD44v are necessary for tumour spread and metastasis, and their expression is generally associated with an unfavourable prognosis[7–16] in DLBCL and in several other haematological malignancies, such as multiple myeloma, NHL, Hodgkin lymphoma, and acute myelogenous leukaemia.[13–24]

> "CD44 isoforms play a key role in lymphocyte migration, homing, and activation and participate in the transmission of signals that regulate haemopoiesis and apoptosis"

With respect to CD44 expression in DLBCL, previous studies largely failed to take into account the clinicopathological heterogeneity of this disease and distinguished cases based on the CD44 expression profile alone. Therefore, we analysed the expression and prognostic significance of CD44 and its variant isoforms CD44v4, CD44v6, and CD44v9 on the background of the recently identified ABC and GC subgroups of DLBCL using a lymphoma tissue microarray (TMA) comprising 90 immunophenotypically profiled DLBCLs with complete clinical follow up. TMA technology allows simultaneous morphological and immunohistochemical analysis of up to 1000 different specimens on a single slide under identical processing conditions.[25 26] Recently, we and others demonstrated that it is also a reliable and highly effective method for in situ lymphoma studies.[2 27–31]

MATERIALS AND METHODS

Patients

Ninety formalin fixed, paraffin wax embedded DLBCL tissue samples from the archives of the institute of pathology at the University of Bologna (Italy) were included in our study. They consisted of 82 previously untreated, newly diagnosed de novo

Abbreviations: ABC, activated B cell-like; CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; DLBCL, diffuse large B cell lymphoma; FFS, failure free survival; FL, follicular lymphoma; GC, germinal centre B cell-like; H&E, haematoxylin and eosin; IPI, international prognostic index; NHL, non-Hodgkin lymphoma; OS, overall cumulative survival; SLL, small lymphocytic lymphoma; TMA, tissue microarray

Table 1 Antibodies and antigen retrieval techniques used

Antibody/antigen	Dilution	Retrieval	Source
CD10	1/10	Pressure cooker, 121°C, 5 minutes	Dako, Glostrup, Denmark
CD20	1/700	Microwave oven, 800 W, 10 minutes	Dako
bcl-2	1/50	Pressure cooker, 121°C, 5 minutes	Dako
bcl-6	1/40	Pressure cooker, 121°C, 5 minutes	Dako
CD44s	1/10	Microwave oven, 800 W, 60 minutes	*
CD44v4	1/10	Microwave oven, 800 W, 60 minutes	*
CD44v6	1/10	Microwave oven, 800 W, 60 minutes	*
CD44v9	1/10	Microwave oven, 800 W, 60 minutes	*

*Monoclonal antibodies produced and kindly given by Dr U Günthert.
CD44s, CD44 standard isoform; CD44v, CD44 variant isoform.

DLBCLs, six cases of transformed follicular lymphoma (FL), and two cases of transformed small lymphocytic lymphoma (SLL). Follow up data of 71 patients (36 women and 35 men) aged between 29 and 90 years (mean, 62.1) were available. According to the Ann-Arbor classification, 12 cases were at clinical stage I, 15 at stage II, 15 at stage III, and 21 at stage IV. Twenty one patients had an international prognostic index (IPI) of 0, 14 had an IPI of 1, 13 had an IPI of 2, nine had an IPI of 3, three had an IPI of 4, and one had an IPI of 5. Disease remission was defined as absence of disease for at least one month after cessation of the last treatment regimen, as assessed by laboratory and imaging studies and physical examination. Disease relapses were defined as disease recurring at least one month after disease remission. Freedom from treatment failure was defined as lack of relapses and treatment resistance. Twelve relapses occurred. Twenty three patients died as a result of treatment failure and two because of cardiovascular disease. Within the follow up period of one to 130 months (mean, 27), cumulative overall survival (OS) was 49.5%.

Tissue microarray construction

For TMA construction, a haematoxylin and eosin (H&E) stained slide was made from each block and reviewed by at least two expert haematopathologists. Representative tumour 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 incorporated into a recipient paraffin block using a precision instrument (Beecher Instruments, Silver Spring, Maryland, USA), as described previously.[25 26] To overcome the problem of tissue microheterogeneity and to increase the number of evaluable cases, each donor tissue block was punched three times for the construction of the recipient block, so that each block contained 270 tissue cores. Sections (4 μm thick) of these TMA blocks were transferred to an adhesive coated glass slide system (Instrumedics Inc, Hackensack, New Jersey, USA).

Immunohistochemistry

Table 1 lists the primary antibodies against CD10, CD20, bcl-2, bcl-6, CD44s, CD44v4, CD44v6, and CD44v9, their dilutions, and the pretreatment conditions. Bound secondary antibodies were visualised by standard avidin–biotin–peroxidase techniques using diaminobenzidine as chromogen. Because weak staining for CD44s was consistently present in up to 20% of the small reactive background lymphocytes, similar staining patterns in lymphomas were not considered to be specific tumour associated CD44s expression; membranous staining in 20–70% of the malignant cells was considered positive, and in over 70% as strongly positive. CD44v staining in 10–20% of the malignant cells was considered weakly positive, in 20–70% as positive, and in over 70% as strongly positive.[16] Positive cases were analysed together by the Spearman rank test and the Kaplan-Meier method. Normal tonsils were used as positive controls, and for negative controls the primary antibodies were omitted.

Statistical analysis

Statistical analysis was performed using the statistical package of social sciences (SPSS). Failure free survival (FFS) and OS were analysed by the Kaplan-Meier method and compared by the log rank test. The Spearman rank test and Fisher's exact test were applied to demonstrate correlations. Multivariate analysis for the prognostic effect of the expression of CD44 and its variant isoforms (CD44v4, CD44v6, and CD44v9), age, sex, Ann-Arbor stage, IPI, bcl-2, bcl-6, and CD10 was performed using a general linear model. p Values below 0.05 were considered significant.

RESULTS

Histopathology and immunohistochemistry

All 90 cases tested were representative by H&E morphology. There was no tissue at 21 positions of the TMA, and at two positions no tumour was seen. However, each sample was punched three times; therefore, at least one evaluable core was present in almost all cases. All examined samples showed strong expression of CD20; 26 of the 90 cases were also positive for CD10, 52 of 89 for bcl-2, and 38 of 90 for bcl-6 (table 2; fig 1).

To validate the TMA approach for our study, we compared the expression of CD10, CD20, bcl-2, and bcl-6 with the results obtained on conventional full tissue sections of 60 cases and found a concordance of 100% for each marker. According to the immunohistochemically detectable expression of bcl-2, bcl-6, and CD10, the analysed DLBCLs were tentatively subclassified into GC type DLBCL (bcl-6+/CD10+/bcl-2–; 22 cases) and ABC type DLBCL (bcl-6–/CD10–/bcl-2+; 25 cases), in addition to unclassifiable cases (all other expression profiles; 35 cases).[3–6 32]

Because of factors related to the TMA technology,[27] nine (CD44s), seven (CD44v4, CD44v6), and three (CD44v9) cases of de novo DLBCL contained no tissue on the slides and, therefore, could not be evaluated. Table 3 summarises the expression profile of CD44s and CD44v in our study group. The staining pattern was intense and confined to the cell membrane, with a characteristic submembranous rim (fig 2).

Table 2 Tissue microarray immunoprofiling of de novo and secondary diffuse large B cell lymphomas (DLBCLs)

Lymphoma subtype	CD10	CD20	bcl-2	bcl-6
De novo DLBCL	22/82	78/78	45/81	35/82
Transformed FL	4/6	6/6	5/6	3/6
Transformed SLL	0/2	2/2	2/2	0/2

FL, follicular lymphoma; SLL, small lymphocytic lymphoma.

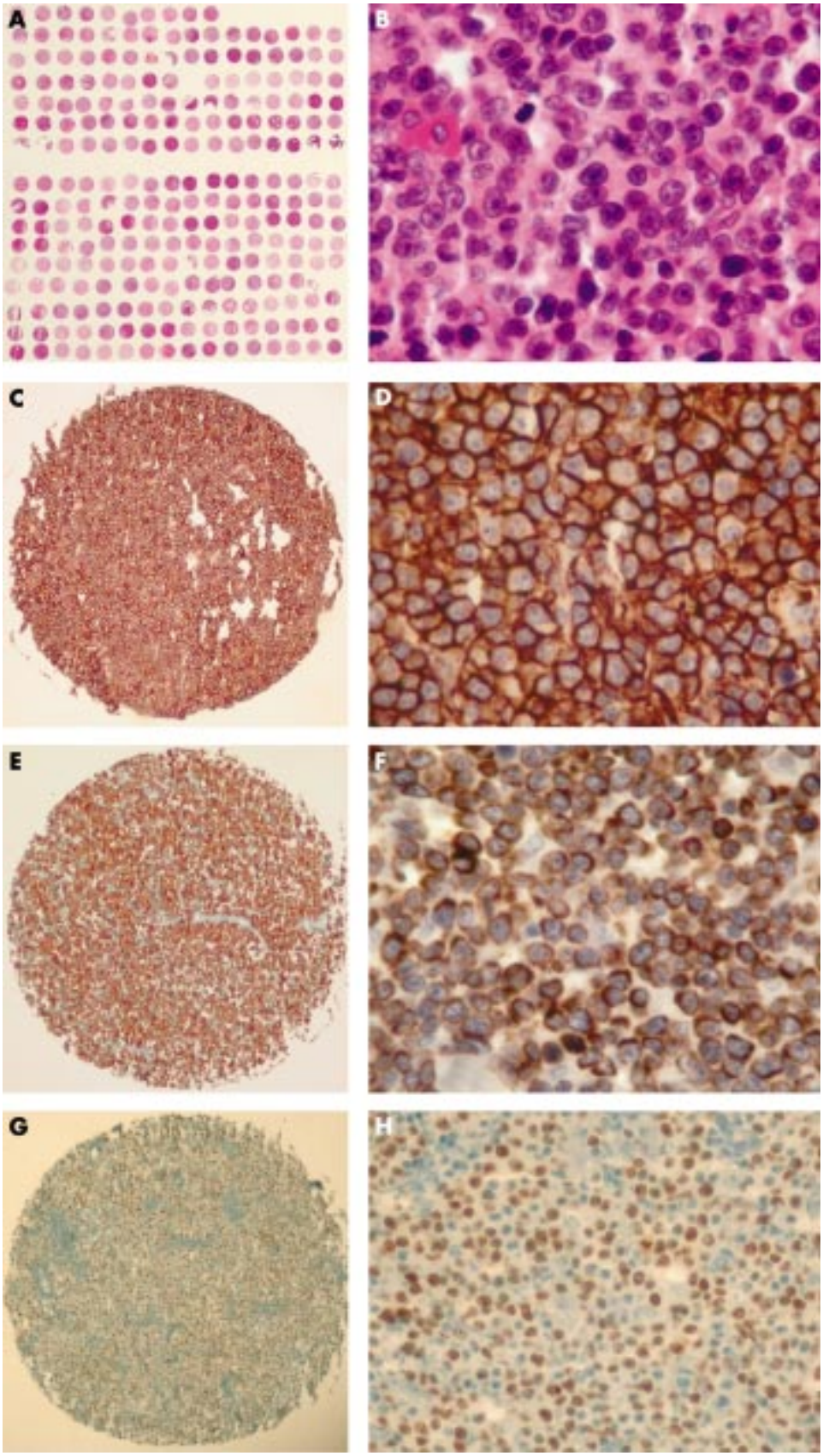

Figure 1 Diffuse large B cell lymphoma (DLBCL) tissue microarray (TMA). (A) Overview of the lymphoma TMA. Haematoxylin and eosin (H&E) stain; original magnification, ×2. (B) DLBCL TMA, high magnification. H&E stain; original magnification, ×400. (C) CD20 expression in a tissue core of DLBCL. Immunoperoxidase stain; original magnification, ×20. (D) CD20 expression in a tissue core of DLBCL. Immunoperoxidase stain; original magnification, ×400. (E) Bcl-2 expression in a tissue core of DLBCL. Immunoperoxidase stain; original magnification, ×20. (F) Bcl-2 expression in a tissue core of DLBCL. Immunoperoxidase stain; original magnification, ×400. (G) Bcl-6 expression in a tissue core of DLBCL. Immunoperoxidase stain; original magnification, ×20. (H) Bcl-2 expression in a tissue core of DLBCL. Immunoperoxidase stain; original magnification, ×200. Note the excellent representativity of all TMA cores shown

Reactive background cells expressed none of the examined CD44v isoforms. Only two de novo DLBCLs expressed CD44v4.

Statistics

In primary DLBCL, univariate analysis showed that CD10 and bcl-6 were coexpressed (22 of 35 bcl-6 positive cases also expressed CD10; $p = 0.005$), whereas there was an inverse correlation between the distribution of bcl-2 and bcl-6 (13 of 45 bcl-2 positive cases expressed bcl-6; $p < 0.05$). CD44v6 was colocalised with bcl-2 ($p < 0.05$), and appeared predominantly on bcl-6 negative cases ($p = 0.05$). Applying Fisher's exact test, OS correlated positively with the expression of CD10, bcl-6, and CD44s ($p < 0.05$), and inversely with IPI, disease stage, and relapse rate ($p < 0.001$). FFS correlated positively with the expression of CD44s and inversely with IPI,

Table 3 Tissue microarray analysis of the expression of CD44 standard isoform (CD44s) and its variant isoforms CD44v4, CD44v6, and CD44v9 in diffuse large B cell lymphoma (DLBCL)

	CD44s			CD44v6			CD44v9
Lymphoma subtype	10–20%	20–70%	>70%	10–20%	20–70%	>70%	>70%
All de novo DLBCLs	12/73	19/73	28/73	14/75	5/75	4/75	15/79
DLBCL ABC type	8/23	5/23	10/23	5/22	2/22	2/22	4/22
DLBCL GC type	4/22	8/22	7/22	2/22	2/22	0/22	7/22
Transformed FL	0/6	1/6	3/6	0/6	0/6	0/6	0/6
Transformed SLL	1/2	1/2	0/2	0/2	1/2	0/2	1/2

Positive cases out of evaluable cases are shown.
ABC, activated B cell-like; FL, follicular lymphoma; GC, germinal centre B cell-like; SLL, small lymphocytic lymphoma.

disease stage, and relapse rate. The GC type of DLBCL presented with a mean IPI score of 0.6, tumours of the ABC type with 1, and the unclassified group with 1.88 ($p = 0.001$). OS in the CD10 positive group at 60 months after diagnosis was 80%, compared with 48% in the CD10 negative group, 48% in bcl-2 expressing cases, 70% in bcl-2 negative cases, 72% in bcl-6 positive cases, and 46% in bcl-6 negative cases, although these differences were not significant in the log rank test. The OS of patients with GC type DLBCL at 30 months after diagnosis was 89%, compared with 58% in the unclassified group, and 44% in the ABC type (fig 3). Multivariate analysis revealed disease stage to be an independent prognostic factor

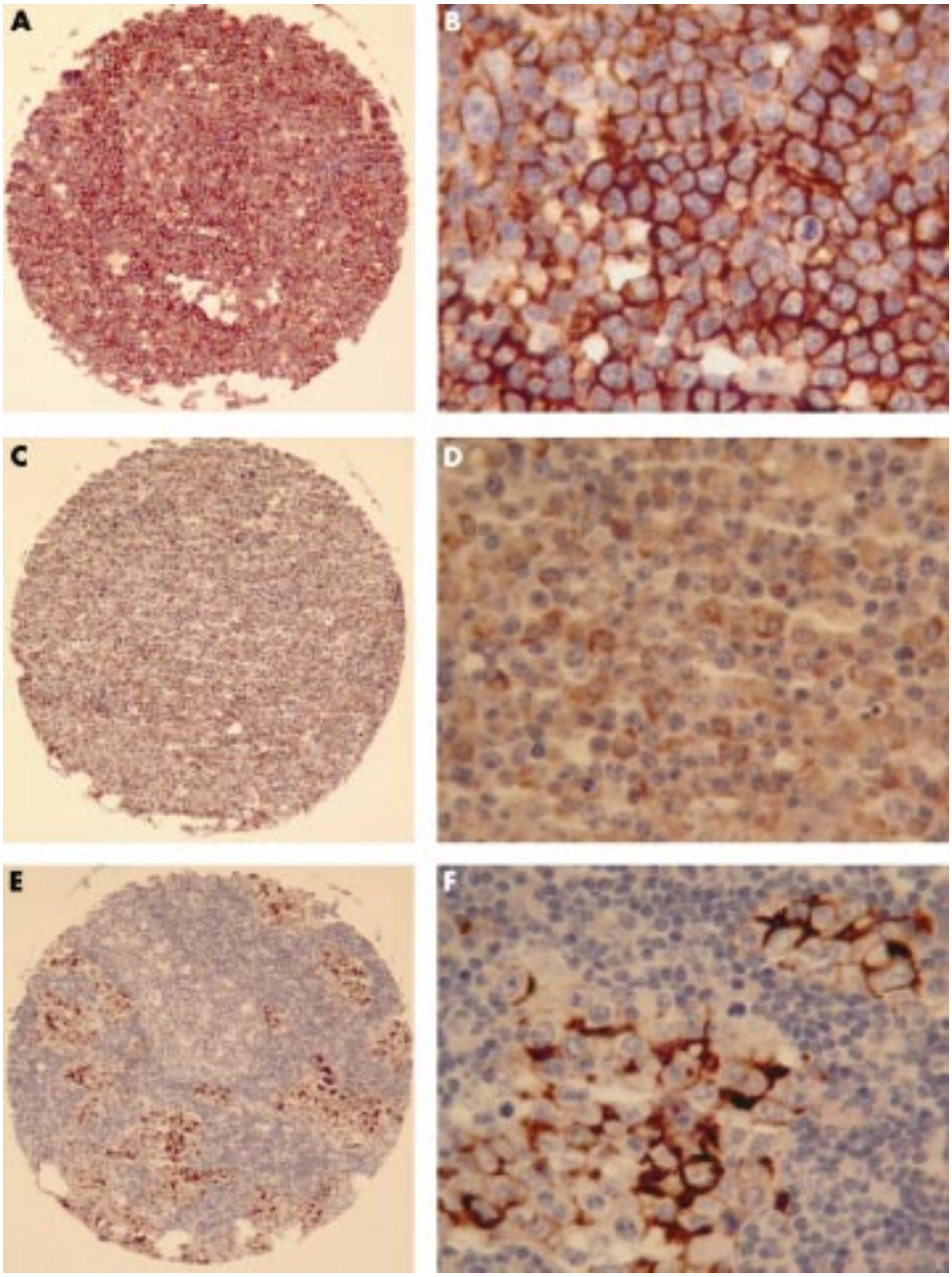

Figure 2 Tissue microarray (TMA) analysis of CD44 expression in diffuse large B cell lymphoma (DLBCL). (A) CD44 standard isoform (CD44s) expression in DLBCL. Immunoperoxidase stain; original magnification, ×20. (B) CD44s expression in DLBCL. Immunoperoxidase stain; original magnification, ×400. (C) CD44 variant isoform 6 (CD44v6) expression in DLBCL. Immunoperoxidase stain; original magnification, ×20. (D) CD44v6 expression in DLBCL. Note the weaker staining intensity compared with CD44s; the background lymphocytes remain unstained. Immunoperoxidase stain; original magnification, ×400. (E) CD44v9 expression in a focal infiltrate of a DLBCL. Even small lymphoma foci can be identified on a TMA core. Immunoperoxidase stain; original magnification, ×20. (F) CD44v9 expression in a focal infiltrate of a DLBCL. Immunoperoxidase stain; original magnification, ×400.

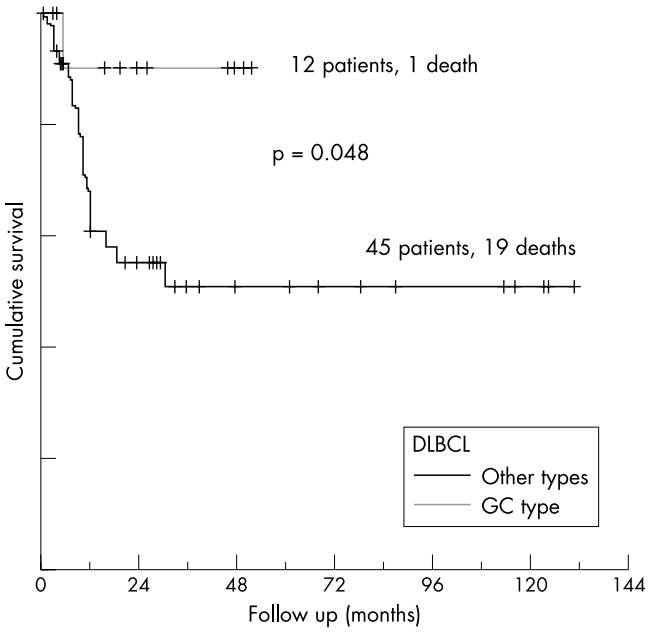

Figure 3 Overall survival in diffuse large B cell lymphoma (DLBCL) correlates with the coexpression of bcl-6 and CD10 (germinal centre (GC) B cell phenotype).

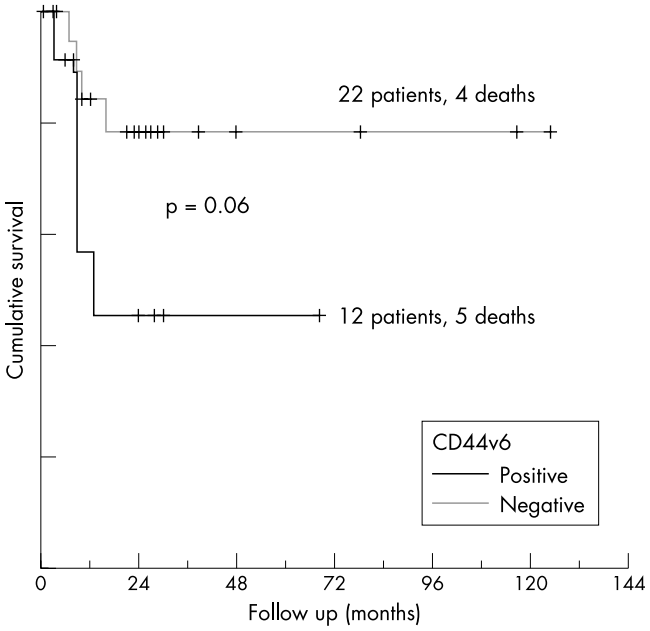

Figure 4 Overall survival in CD44 standard isoform negative diffuse large B cell lymphomas with respect to expression of CD44 variant isoform 6 (CD44v6).

for OS and FFS, as was CD10 and bcl-6 coexpression for OS ($p < 0.05$). OS and FFS correlated with CD44s expression (14 treatment failures in 19 CD44s negative cases compared with 12 in 34 CD44s positive cases; $p < 0.005$). Disease stage and IPI correlated inversely with CD44s expression, whereas disease stage showed a linear correlation with CD44v6 expression ($p < 0.05$). CD44v6 expression separated cases negative for CD44s into two prognostically different groups: the CD44v6 negative group had an OS of 82% versus 58% in the CD44v6 positive group at 70 months after diagnosis ($p = 0.06$) (fig 4). CD44v9 expression had no prognostic value in this study group.

DISCUSSION

We report the construction and validation of a DLBCL TMA containing 270 cores of 90 different samples. This lymphoma array was used to analyse the expression and prognostic significance of CD44. Application of the TMA technology enabled us to explore CD44 protein expression in a higher number of tumour specimens than was possible in previous studies.[13 21] To assess the accuracy of the TMA technology for immunohistochemical analysis, we directly compared the expression of CD10, CD20, bcl-2, and bcl-6 with the results obtained on the corresponding conventional full sections of 60 cases, and found a concordance of 100% for each marker if three cores/case were used. The absence of a single discordant case, similar to a recent TMA study on Hodgkin lymphoma,[27 31] demonstrates that the TMA methodology is very useful for clinicopathological investigations of large lymphoma series. A similarly high degree of correlation (86–100%) for nine commonly used lymphoma markers was recently reported in a lymphoma TMA validation study using two cores/case.[30]

The immunophenotypical profile of the tumours showed a clear segregation of the de novo DLBCLs into a bcl-2–/bcl-6+/CD10+ (GC type; 22 cases) and a bcl-2+/bcl-6–/CD10– group (ABC type; 25 cases), in addition to a third group showing coexpression or lack of expression of these three markers (35 cases). Coexpression of CD10 and bcl-6 was, along with disease stage, of independent prognostic significance for OS. The OS of patients with GC type DLBCL at 30 months after diagnosis was 89%, compared with 58% in the unclassified cases and 44% in ABC type DLBCL. Our results confirm and extend recent observations using cDNA and oligonucleotide microarrays,[2–6] indicating that de novo DLBCLs consist of at least three histogenetically, phenotypically, and prognostically different entities. Thus, bcl-2, bcl-6, and CD10 might be useful as surrogate markers for the complex gene expression pattern of the molecularly defined DLBCL subtypes.[32]

> "Disease stage correlated inversely with CD44 standard isoform expression and showed a linear correlation with CD44 variant isoform 6 expression"

We observed a complex expression pattern of CD44s and its variant isoforms CD44v4, CD44v6, and CD44v9 in DLBCL. Whereas de novo DLBCLs expressed CD44s and all variants, transformed FLs, similar to their normal counterparts,[33] expressed only CD44s and no variant isoforms. Of particular interest was CD44v expression in the tentative GC and ABC subgroups of de novo DLBCL. Univariate analysis showed that CD44v6 was predominantly expressed in bcl-2 positive cases (major marker of activated B cells), but not in bcl-6 positive tumours (major marker of germinal centre B cells). This distribution of CD44v6 corresponds to the gene expression profile of ABC type DLBCL.[3 6] Importantly, CD44v6 expression is considered to be a signature of activated B cells because it is upregulated in peripheral blood B cells upon activation.[6 7 10 33]

Disease stage correlated inversely with CD44s expression and showed a linear correlation with CD44v6 expression. These results support the concept that the expression of CD44v6 might contribute to lymphoma dissemination.[8 12] In our study group, CD44v6 expression per se was not prognostically significant, but in CD44s positive cases, it played an important albeit minor role ($p = 0.06$), as suggested in many previous studies.[13–15 18 21] In CD44s negative cases, CD44v6 positive lymphomas had a worse OS. Furthermore, CD44v6 was expressed predominantly in advanced stage disease and in DLBCLs of the ABC type. The expression of CD44v6 in CD44s positive and CD44s weakly positive cases had no prognostic impact, perhaps because of the accumulation of unclassifiable DLBCL cases in this group. This could also explain the better clinical outcome observed in the CD44s positive group. Whether these prognostic differences result from direct CD44v6 mediated effects, such as lymphoma spread and increased resistance to chemotherapy induced apoptosis, or

Take home messages

- Tissue microarray technology is useful for immunophenotyping and the clinicopathological analysis of large lymphoma populations and correlated 100% with the results from conventional analysis
- Overall survival (OS) was significantly better in those patients with the germinal centre B cell-like phenotype (bcl-2–/bcl-6+/CD10+) of diffuse large B cell lymphoma (DLBCL)
- Expression of CD44 variant isoform 6 (CD44v6) correlates with disease stage, and might contribute to lymphoma dissemination
- CD44v6 was expressed predominantly in activated B cell-like (ABC) DLBCL (bcl-2+/bcl-6–/CD10–), and in CD44 negative cases it was associated with worse OS
- CD44v6 might be useful in conjunction with bcl-2, bcl-6, and CD10 to help subclassify DLBCL cases into ABC and GC types, and this might be of practical importance for lymphoma diagnosis and individualised treatment

CD44v6 expression is only a bystander signature of the different genetically defined DLBCLs, remains to be determined. Nonetheless, searching for additional immunohistochemical markers, such as CD44v6, which can be used in conjunction with bcl-2, bcl-6, and CD10 to help subclassify DLBCL cases into ABC and GC types might be of practical importance for lymphoma diagnosis and individualised treatment.

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