Expression of PKC-beta or cyclin D2 predicts for inferior survival in diffuse large B-cell lymphoma

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We sought to determine whether identification of poor-risk subgroups of diffuse large B-cell lymphoma (DLBCL) using immunohistochemical stains would have practical utility with regard to prognosis and therapeutic decisions. Tissue microarray blocks were created using replicate samples of formalin-fixed, paraffin-embedded tissue from 200 cases of *de novo* DLBCL. The sections were stained with antibodies to proteins that are expressed by activated or proliferating B cells including MUM1, FOXP1, bcl-2, survivin, protein kinase C-beta (PKC- β), cyclin D2, cyclin D3, and Ki-67. In univariate analysis, tumor expression of cyclin D2 ($P=0.025$) or PKC- β ($P=0.015$) was associated with a worse overall survival, whereas none of the other markers was predictive of overall survival. Patients with DLBCL that expressed either cyclin D2 or PKC- β had a 5-year overall survival of only 30% as compared to 52% for those who were negative for both markers ($P=0.0019$). In multivariate analysis, the expression of cyclin D2 or PKC- β was an independent predictor of poor overall survival ($P=0.035$). Cyclin D2 and PKC- β expression will be useful in designing a 'biological prognostic index' for patients with DLBCL.

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Diffuse large B-cell lymphoma (DLBCL) accounts for 30–40% of all new diagnoses of non-Hodgkin's lymphoma.^{1,2} However, durable remissions are achieved in only 40–50% of patients with DLBCL despite the use of anthracycline-based chemotherapy.² The addition of rituximab to standard chemotherapy appears to improve the survival of patients with DLBCL expressing bcl-2.³ The prognosis of patients with DLBCL is currently estimated using the clinical parameters of the International Prognostic Index (IPI), but these clinical parameters are likely to be surrogates for underlying biological differences.⁴ Therefore, it is important to find biological markers at the time of diagnosis that

will identify patients who may benefit from more aggressive or experimental therapies.

Recent studies have demonstrated that DLBCL can be divided into prognostically important subgroups by gene expression profiling using cDNA or oligonucleotide microarrays.^{5–9} Four subgroups, identified as germinal center B-cell-like, activated B-cell-like, primary mediastinal, and unclassified DLBCL have been delineated using cDNA microarrays.^{5,7,8,10} The germinal center B-cell-like and primary mediastinal subgroups are reported to have a significantly better survival than the activated B-cell-like and unclassified subgroups.^{5,7,8,10} Alternatively, DLBCL can be divided into two molecularly distinct subgroups of cured and fatal/refractory DLBCL.⁶ Since this microarray technology is expensive and not generally available, a simpler and more widely available method to identify those patients with aggressive or refractory disease would have wide applicability and practical utility in routine clinical practice. The goal of this study was to identify poor-risk subgroups of DLBCL using immunohistochemical stains for biologic markers that may be associated with aggressive disease.

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Patients and methods

We studied 200 adult patients in the Nebraska Lymphoma Study Group Registry with *de novo* DLBCL, available archival tissue, and at least 1 year of follow-up for surviving patients. The median follow-up of the surviving patients was 7.7 years (range, 1.3–19.6 years). Each patient was evaluated and staged according to a standard protocol and all patients were treated with curative intent. Therapy for 110 patients (55%) consisted of combination chemotherapy including cyclophosphamide, doxorubicin or mitoxantrone, procarbazine, bleomycin, vincristine, and prednisone or dexamethasone (CAP-BOP),¹¹ whereas 85 patients (42%) received cyclophosphamide, mitoxantrone, vincristine, prednisone (CNOP),¹² and five patients (3%) received radiation therapy for localized disease. No patient received rituximab as part of the initial chemotherapy regimen. The University of Nebraska Medical Center Institutional Review Board approved this study and informed consent was obtained in all cases.

To evaluate biological markers, we created tissue microarrays containing representative tissue from each case. To create each tissue microarray, hematoxylin and eosin-stained sections from each paraffin-embedded, formalin-fixed block were reviewed to define diagnostic areas. From these diagnostic areas, four random, representative 0.6 mm cores were obtained from each case and inserted in a grid pattern into a recipient paraffin block using a tissue arrayer (Beecher Instruments, Silver Spring, MD, USA). Five micrometer sections were cut from each tissue microarray and stained with antibodies to MUM1, FOXP1, bcl-2, survivin, protein kinase C- β (PKC- β), cyclin D2, cyclin D3, and Ki-67 (Table 1). These antibodies were selected because they are applicable to paraffin-embedded tissue and the corresponding proteins are expressed by activated or proliferating B cells, including some proteins associated with the activated B-cell-like gene expression signature.^{5,7} Following deparaffinization, heat-induced antigen retrieval techniques were employed for each antibody (Table 1). An amplification kit (Ventana Medical Systems, Tucson, AZ, USA) was used to enhance staining for FOXP1, survivin, PKC- β and cyclin D3. Following antigen retrieval and primary antibody incubation, the reaction was completed in a Ventana ES instrument using a diaminobenzidine immunoperoxidase detection kit (Ventana). A CD20 stain was also performed to evaluate each core for involvement by tumor. Each core was evaluated by visual estimation for the percentage of tumor cells positive with each antibody and the findings were recorded in 10% increments. For each case, the core with the highest percentage of tumor cells stained was used for analysis. A survival tree method was used to evaluate all possible cut-points for statistical significance.¹³ The intensity of staining was not used in

Table 1 Antibodies used for immunohistochemical staining of DLBCL

Antibody	Clone	Source	Antigen retrieval	Dilution
CD20	L26	Dako, Carpinteria, CA, USA	Citrate 30	1:200
MUM1	MUM1p ^a	Falini ⁽⁵⁸⁾	EDTA 30	1:10
FOXP1	JC12	Banham ⁽⁵⁹⁾	EDTA 30	1:80
Bcl-2	124	Dako, Carpinteria, CA, USA	EDTA 30	1:10
Survivin	Polyclonal	Novus Biologicals, Littleton, CO, USA	Citrate 10	1:500
PKC- β	28	Serotec, Oxford, UK	EDTA 30	1:75
Cyclin D2	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	EDTA 30	1:500
Cyclin D3	DCS-22	NeoMarkers, Fremont, CA, USA	Citrate 10	1:100
Ki-67	Polyclonal	Dako, Carpinteria, CA, USA	Citrate 60	1:20

Citrate 10 indicates 10 min at 114°C in a pressure cooker in citrate (10 mM, pH 6.0), Citrate 30 indicates 30 min at 95°C in citrate (10 mM, pH 6.0), Citrate 60 indicates 60 min at 95°C in citrate (10 mM, pH 6.0), EDTA 30 indicates 30 min at 95°C in EDTA (1 mM, pH 8.0).

^aThis clone is now commercially available from Dako.

the determination of positivity since differences in fixation and processing may result in variable staining. Cases were considered positive if 30% or more of the tumor cells were stained, except for PKC- β and Ki-67 for which a cutoff of greater than 50% was used. As the survival tree method did not yield more informative cutoffs, the 30% cut-point was chosen based on a prior analysis of some of the markers.^{14,15} In addition, others have used similar cutoffs with tissue microarrays or whole sections.^{16,17} A cutoff of greater than 50% was chosen for PKC- β because this value was found to be the most informative using the survival tree method. There is no consensus in the literature regarding the best cut-point for Ki-67 and the survival tree method did not yield an informative cut-point; therefore, we chose 50%, which was the mean Ki-67 expression in our study population.

Fisher's exact test was used to compare the clinical characteristics of the patients. Median ages were compared using the Wilcoxon rank-sum test. The Kaplan–Meier method was used to estimate overall and event-free survival distributions.¹⁸ Overall survival was calculated as the time from diagnosis to the date of death or last contact. Patients who were alive at last contact were treated as censored for overall survival analysis. Event-free survival was calculated from the time of diagnosis to the date of progression, death, or last contact. Patients who were alive at last contact and had not progressed were treated as censored for event-free survival analysis. The log-rank test was used to compare survival distributions. Multivariate analysis was performed using the Cox regression method.¹⁹ Stepwise selection was employed to determine

the variables that were independent predictors of overall survival using a significance level of $P=0.05$. SAS software (SAS Institute Inc, Cary, NC, USA) was employed for the data analysis.

Results

Clinical data were available for all patients. The patients consisted of 94 men and 106 women with a median age of 69 years (range, 19–93 years). The median follow-up of the surviving patients was 7.7 years (range, 1.3–19.6 years). The 5-year overall survival for the entire group was 44% and the 5-year event-free survival was 35%.

Expression of cyclin D2 was seen in 19% (38/200) of the cases, PKC- β in 22% (41/187), MUM1 in 45% (89/200), bcl-2 in 58% (115/200), FOXP1 in 61% (119/196), Ki-67 in 62% (119/193), cyclin D3 in 62% (122/197), and survivin in 65% (125/193). Univariate analysis of the expression of each protein and its relationship to overall survival and event-free survival was performed. Tumor expression of cyclin D2 (Figure 1a) was associated with a significantly shorter overall survival ($P=0.025$; Figure 2a) and event-free survival ($P=0.07$). Similarly, PKC- β expression (Figure 1b) predicted for worse overall survival ($P=0.015$; Figure 2b) and event-free survival ($P=0.04$). Cases with tumor expression of MUM1 demonstrated a trend toward worse overall survival, but this did not reach statistical significance ($P=0.18$). None of the other proteins were predictive of overall survival or event-free survival.

There was little overlap in the expression of cyclin D2 and PKC- β in DLBCL. Of the 71 cases positive for either cyclin D2 or PKC- β , only eight cases were positive for both markers. Therefore, we combined the results of these two stains to determine survival outcomes. The combination of either PKC- β or cyclin D2 positivity predicted for significantly worse overall survival ($P=0.002$; Figure 3a) and event-free survival ($P=0.012$; Figure 3b). All eight patients expressing both cyclin D2 and PKC- β died with a median overall survival of only 16 months, which is similar to the median overall survival of 15 months for those expressing either cyclin D2 or PKC- β . A comparison of the clinical characteristics of cases expressing cyclin D2 or PKC- β to those who were negative for both markers is shown in Table 2. There were no significant differences in IPI scores or the components that comprise the IPI score between these two groups. However, patients who were positive for PKC- β or cyclin D2 were more likely to have B symptoms than those who were negative for both markers. When evaluating the expression of PKC- β or cyclin D2 within the context of IPI scores, these two markers added prognostic value to the IPI score. Patients with low IPI scores (0–2) who expressed PKC- β or cyclin D2 had a significantly worse overall survival ($P=0.037$, Figure 4a) compared to those lacking

Figure 1 Immunoperoxidase staining. (a) Nuclear expression of cyclin D2 ($\times 100$); (b) cytoplasmic expression of PKC- β ($\times 100$).

both markers. A similar trend was observed for patients with high IPI scores (3–5) who expressed PKC- β or cyclin D2 ($P=0.10$, Figure 4b), however, this did not reach statistical significance.

Multivariate analysis was performed and stepwise selection was employed to determine the variables that were independent predictors of overall survival. Variables considered in the analysis were the IPI risk groups (0–2 and 3–5), the expression of PKC- β or cyclin D2, and expression of each of the other proteins studied. In addition to a high IPI score, the expression of PKC- β or cyclin D2 was a significant independent predictor of worse overall survival. The risk of death was 2.6 times (95% CI, 1.8–3.7)

Figure 2 Expression of cyclin D2 and PKC- β in DLBCL. (a) Cyclin D2 expression and overall survival; (b) PKC- β expression and overall survival.

greater for patients with a high IPI score ($P < 0.0001$) and 1.6 times (95% CI, 1.1–2.3) greater for those expressing PKC- β or cyclin D2 ($P = 0.018$).

Discussion

The identification at the time of diagnosis of patients with DLBCL who are likely to have a poor prognosis could have an important impact on therapeutic decisions. The use of aggressive or experimental treatments might lead to better outcomes for this group of patients. Although prognostic subgroups of DLBCL have been identified using cDNA or oligonucleotide microarrays, this technology is currently not available for routine clinical use.^{5–7} In addition, this method requires the use of fresh or frozen tumor tissue, which is often not

Figure 3 Expression of either PKC- β or cyclin D2 (CyD2) in DLBCL. (a) Relationship to overall survival; (b) relationship to event-free survival.

available. On the other hand, immunohistochemical stains are routinely used by pathologists for diagnosis. Therefore, the use of immunohistochemical stains to identify patients with a poor prognosis would have practical utility for the initial management of patients with DLBCL.

Using a panel of immunostains, we sought to identify a group of patients with DLBCL who had poor overall survival and event-free survival. Our selection of the individual antibodies was based on data derived from gene expression studies^{5–7} and reports in the literature.^{20–22} We found that tumor expression of PKC- β or cyclin D2 was significantly associated with a poor outcome in DLBCL. Patients expressing either marker had a median overall survival of only 15 months as compared to 65

Table 2 Clinical features of patients with DLBCL expressing cyclin D2 or PKC- β (positive) compared to those lacking both markers (negative)

	Total ^a	Positive	Negative	P value
Total number	200	71	118	
Sex				
Male	94 (47%)	36 (51%)	55 (47%)	0.65
Female	106 (53%)	35 (49%)	63 (53%)	
Age (years)				
Median	69	69	69	0.07 ^b
Range	19–93	19–92	19–93	
Stage				
I/II	114 (58%)	36 (51%)	71 (61%)	0.23
III/IV	84 (42%)	34 (49%)	46 (39%)	
Extranodal sites				
Fewer than two	152 (77%)	52 (74%)	92 (79%)	0.59
Two or more	46 (23%)	18 (26%)	25 (21%)	
Karnofsky score				
Higher than 70	161 (81%)	55 (79%)	96 (81%)	0.71
70 or lower	38 (19%)	15 (21%)	22 (19%)	
LDH				
Normal	108 (56%)	37 (55%)	64 (56%)	1.00
High	84 (44%)	30 (45%)	51 (44%)	
B symptoms				
No	142 (72%)	43 (62%)	90 (77%)	0.04
Yes	55 (28%)	26 (38%)	27 (23%)	
IPI risk group				
Low (0–2)	126 (66%)	39 (58%)	79 (69%)	0.15
High (3–5)	65 (34%)	28 (42%)	35 (31%)	

LDH, serum lactate dehydrogenase; IPI, International Prognostic Index.

^aPKC- β staining was evaluable in 187 cases; an additional two cases were positive for cyclin D2 but not evaluable for PKC- β .

^bAlthough the median age and age range are nearly identical, the age distribution of the negative group showed a slightly older tendency.

months for those lacking both markers (Figure 2a). Furthermore, when used together, expression of either cyclin D2 or PKC- β is an independent predictor of worse overall survival.

Cyclin D2 is a cell cycle regulatory protein that facilitates the G1 to S phase transition. By immunohistochemistry, cyclin D2 expression has been reported to occur in 14% of DLBCL.²³ Similarly, we found that 19% of our cases were positive for cyclin D2. In the current study, we also found that expression of cyclin D2 was associated with a significantly worse overall survival ($P = 0.025$). We were the first to report, in a different group of patients with DLBCL and gene expression profiling data, that cyclin D2 expression predicts for worse survival in DLBCL and that it is limited to DLBCL with an activated B-cell-like or unclassified gene expression profile.²³ No other studies evaluating the prognostic significance of cyclin D2 expression in DLBCL have been published.

Figure 4 Expression of either PKC- β or cyclin D2 (CyD2) within IPI groups in DLBCL. (a) Overall survival of patients with low IPI scores (0–2); (b) overall survival of patients with high IPI scores (3–5).

We also studied the expression of cyclin D3, another member of this gene family, but found that it had no effect on survival in DLBCL. Another study found that aggressive B-cell lymphomas with cyclin D3 expression showed a trend towards worse overall survival, but this did not reach statistical significance.¹⁸ In contrast, a single study has reported that cyclin D3 predicts for worse overall survival in DLBCL.²⁰ Although the cyclin D3-positive cases in that study were significantly more likely to have higher stage (III or IV) disease, more extranodal sites, and higher IPI scores, multivariate analysis showed cyclin D3 expression to be an independent adverse prognostic factor.

PKC- β is a protein kinase that is involved in signal transduction and plays an important role in B-cell receptor signaling.^{24,25} In a group of patients with DLBCL evaluated by an oligonucleotide microarray, PKC- β was one member of a 13 gene predictor of

outcome.⁶ Shipp *et al*⁶ also found that an immunoperoxidase stain for PKC- β correlated with the amount of transcript determined by oligonucleotide microarray analysis. Furthermore, those cases expressing PKC- β by immunohistochemistry had a significantly worse overall survival. However, immunostains for PKC- β were graded similar to the microarray results in that study and, therefore, a quantitative cut-point was not determined. In our study, we used the same monoclonal antibody and our results (22% positive) are comparable to their finding of PKC- β expression in 38% of DLBCL by immunoperoxidase staining. Another immunohistochemical study reported that PKC- β is expressed in 71% of DLBCL.²⁶ However, we found PKC- β expression in greater than 50% of tumor cells in only 22% of our cases. There was no mention of the cut-point used to determine positivity and this study²⁶ used a different monoclonal antibody that was raised against PKC- β from rabbit brain, which may not have the same reactivity as the mouse monoclonal antibody (clone 28) that was used in our study.

None of the other markers that we evaluated were predictive of survival in this group of patients with DLBCL. Numerous studies have been published on these markers and most show conflicting results. Bcl-2 has been studied extensively in DLBCL and the results are controversial. Multiple studies have looked at the expression of bcl-2 using immunostains and most have found no difference in overall survival.^{16,23,27–35} However, some studies have found that bcl-2 expression is associated with a significantly worse overall survival,^{3,36–42} or event-free survival.^{29–31,36,37} The discrepancies related to bcl-2 expression and prognosis may be related to the underlying subgroups of DLBCL. Within the non-germinal center B-cell-like subgroup of DLBCL, bcl-2 expression is associated with a significantly worse overall survival and event-free survival, whereas bcl-2 expression is not predictive of survival within the germinal center B-cell-like subgroup.^{23,43,44} Similarly, MUM1 has been reported to be associated with worse overall survival by some investigators,^{23,43,44} but was not found to be predictive by others.⁴² Uniform high expression of FoxP1 has been associated with a poor outcome.⁴⁵ Although we used a cut-point of 30% for FOXP1, we evaluated all cut-points for significance using a survival tree method and none were significant. In addition, we specifically compared overall survival of those with 100% FOXP1 expression (28% of cases) to those with less than 100% FOXP1 expression. Although there was a trend toward worse overall survival in the 100% FOXP1 group, the difference was not significant ($P=0.16$). Studies on the predictive value of Ki-67 expression are also conflicting and the positive cut-points in DLBCL vary dramatically between studies, ranging from 10 to 80%. Expression of Ki-67 has been associated with significantly worse overall survival,^{31,46–49} significantly better overall survival,^{50–52} or to have no effect on overall survival in

DLBCL.^{16,32,53,54} Although we initially used a cut-point of 50% for Ki-67, we analyzed all possible cut-points using a survival tree method and found no value that had a statistically significant effect on overall survival or event-free survival.¹³ A single study has reported that survivin expression is an independent predictor of worse survival in DLBCL.²¹ Similar to that study, in which 60% of the cases were positive, we found survivin expression in 65% of our group. Despite this similarity, we did not find any prognostic effect of survivin expression in DLBCL.

Given the variability of outcomes in these retrospective studies, it is doubtful that any individual marker can be used consistently to predict survival in DLBCL. In addition, although individual genes may be highly predictive of outcome in gene expression studies, no single gene accurately subclassifies all cases of DLBCL.^{6,7} Therefore, a number of markers need to be combined to form a 'biological prognostic index' which will predict survival in DLBCL, using either gene expression or protein expression methodologies. When used alone or in combination, we found that expression of cyclin D2 or PKC- β identifies a group of patients with significantly worse overall survival and event-free survival. Furthermore, in multivariate analysis, the combination of these two markers was an independent adverse predictor of survival in our patients with DLBCL. Additional studies are needed to confirm our findings. In addition, studies of these markers in DLBCL patients treated with rituximab will be important to evaluate whether rituximab affects outcome. Inhibitors of PKC are being used in clinical trials of patients with relapsed or progressive non-Hodgkin's lymphoma.^{55–57} If our findings are confirmed, the addition of PKC inhibitors to initial chemotherapy regimens should be considered in clinical trials of this group of patients with DLBCL. As additional immunohistochemical markers of aggressive disease are identified through gene expression studies, our findings suggest that cyclin D2 and PKC- β should be included with these new markers to form a 'biological prognostic index' that could be used in clinical trials to stratify patients for risk-adjusted therapies.

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Duality of interest

None declared.

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