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Centroblastic Diffuse Large B Cell Lymphoma Displays Distinct Expression Pattern and Prognostic Role of Apoptosis Resistance Related Proteins

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Centroblastic diffuse large B cell lymphoma (DLBCL) samples were analyzed by immunohistochemistry to evaluate the expression of p53, Bcl-2, Survivin, XIAP, and Ki-67. Survivin was the only protein which expression exhibited a trend for impact in progression-free ($p = .077$) and overall survival ($p = .054$). In the Mann–Whitney test, Survivin expression correlated with a negative overall survival ($p = .045$). These results appeared to be intimately related to Survivin cytoplasmic localization. Moreover, the anti-apoptotic proteins Bcl-2 and Survivin were less frequent in centroblastic DLBCL. Our results indicate that centroblastic DLBCL may be a disease with characteristic biology and clinical course and, therefore, specific prognostic factors.

Keywords: Centroblastic diffuse large B cell lymphoma; p53; Bcl-2; Survivin; XIAP; Ki-67 co-expression; Prognosis; Immunohistochemistry

INTRODUCTION

Diffuse large B-cell lymphomas (DLBCL) are the most common type of high-grade non-Hodgkin lymphoma (NHL), accounting for 30% of NHL. It comprises an aggressive and heterogeneous group of B-cell NHL, which differ in terms of morphology, gene expression profile, and treatment outcome. The most common morphological entities that can be distinguished among DLBCL are the centroblastic and immunoblastic subtypes, which are currently equally treated (1). However, centroblastic DLBCL is not only more common than immunoblastic DLBCL, but is also more susceptible to treatment (2–4). Moreover, centroblastic DLBCL is thought to arise from centroblasts—proliferating germinal center B lymphocytes—and hence, is denoted as centroblastic DLBCL. On the other hand, immunoblastic DLBCL is thought to arise from immunoblasts—positive-selected post-germinal center B lymphocytes—and hence, is termed as immunoblastic DLBCL (5, 6). Owing to the subtypes heterogeneity in terms of clinical course and molecular features, there is a debate on whether these variants should be regarded as different diseases, with adjusted treatment and independent research attention for each subtype.

One of the limitations that has led to the inclusion of both the subtypes in the same category is that the distinction between them is morphological, and therefore, can be subjective. However, perhaps, the main limitation is the absence of conclusive studies on the differences between them with regard to treatment response (7). On the other hand, nowadays, immunohistochemistry represents an important tool in routine services to aid in the discrimination of DLBCL subtypes when the morphology is doubtful (8, 9). Furthermore, several long-term follow-up studies have indeed shown that immunoblastic DLBCL has a worse prognosis than centroblastic DLBCL, although during the first two years after diagnosis, this difference might not be obvious (2–4, 10).

Recently, a correlation between the ABC microarray/immunohistochemistry classification and immunoblastic morphology has been identified (11, 12). Furthermore, at this stage of maturation, the lymphocyte is associated with a prosurvival expression profile (5, 6). However, it has not been possible to correlate the centroblastic subtype to the

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GCB microarray/immunohistochemistry classification (11, 12). In addition, the centroblastic subtype has been characterized by a proliferative gene expression profile (5, 6). On the other hand, it has not been well characterized according to survival-related expression profile. We therefore decided to investigate the expression of apoptosis-related proteins in this subtype of proteins that not only are involved in apoptosis, but have also been reported to impact DLBCL biology, such as Survivin and XIAP; caspase inhibitors, IAPs (Inhibitor of Apoptosis Proteins) (13), Bcl-2, mitochondrial membrane depolarization inhibitor, p53, cell cycle controller, and apoptosis inductor; and ki-67, a proliferation-associated antigen (14).

Moreover, as the immunoblast has a prosurvival profile, the expression of some of these apoptosis resistance related proteins might be associated with this DLBCL variant, which has a worse prognosis than the centroblastic variant. Therefore, when including both the variants in a DLBCL prognostic factor quest, a worse prognosis value of a given apoptosis resistance related protein might actually merely reflect the worse prognosis value of the immunoblastic subtype. Currently, the only parameter used to access DLBCL prognosis is the International Prognostic Index (IPI) (15). Furthermore, therapy provides long-term overall survival of as much as half of the patients (1, 16, 17). Therefore, there is a need to identify and establish biological prognostic factors that can help improve risk stratification and function as targets for new therapies. In the past decade, the expression of several proteins has been studied as possible prognostic biomarkers for DLBCL. The classical candidates are proteins that play crucial roles in the cell cycle (e.g., the cell cycle guardian p53 and the proliferation antigen ki-67 (14)) and apoptotic pathways (e.g., the antiapoptotic members of the Bcl-2 family, such as Bcl-2 itself (14) and the caspase inhibitors, XIAP, and Survivin (13)).

Because of the limited knowledge regarding the expression of apoptosis resistance related proteins in centroblastic DLBCL, we addressed this issue in a group of 81 centroblastic DLBCL cases with the aim of understanding p53, Ki-67, Bcl-2, XIAP, and Survivin expression and features, analyzed using immunohistochemistry, and also evaluated their prognostic value in centroblastic DLBCL.

MATERIAL AND METHODS

Patients' selection

From the 191 adult lymphoma cases registered at the National Cancer Institute (Rio de Janeiro, Brazil) between 1989 and 1993, a total of 81 adult centroblastic DLBCL patients and 6 adult immunoblastic DLBCL patients were enrolled for the present study. They were selected to enter the analysis based on having DLBCL with confirmed morphology, available paraffin-embedded tumor sample(s), and adequate long-term follow-up information. The demographic and clinical data were obtained from the patients' records. Out of the 81 centroblastic DLBCL patients and of the 6 immunoblastic DLBCL patients, 69 and 5, respectively, received anthracycline-based chemotherapy protocols, e.g., CHOP or CNOP. Progression-free survival (PFS) and overall survival (OS) were calculated based on the clinical information retrieved from their medical charts. The PFS and OS were not evaluated in the other 13 patients because their treatment was heterogeneous. The local Institutional Ethic Committee approved this study, which was conducted in accordance with the recommendations of the Helsinki Declaration.

Immunohistochemistry

Tumor samples were collected at diagnostic biopsies. The centroblastic DLBCL diagnosis was confirmed by two pathologists (L.M. Rezende and S.O. Romano) independently and based on standard morphological parameters, following the current WHO classification parameters (18). The immunohistochemical analysis was adopted from a previous work of our group (19). Briefly, 4 μm of formalin-fixed paraffin-embedded sample sections were deparaffinized in xylene and rehydrated in ethanol baths. Bcl-2, XIAP, and Survivin antigenic retrieval was performed in a steamer with a citrate buffer of pH 6.0 for 30 min at 98°C. p53 and Ki-67 antigenic retrieval was performed with the same buffer, but in a pressure cooker for 3 min. Endogenous peroxidase and nonspecific antibody labeling were blocked with 3% hydrogen peroxide and a blocking solution, respectively. Tumor slides were incubated overnight at 4°C with anti-p53 (clone DO-7 Dako), anti-Ki-67 (MIB Dako), anti-Bcl-2 (clone 124 Dako), anti-XIAP (Sigma-Aldrich X4503), or anti-Survivin antibodies (Sigma-Aldrich S8191). As the detection system, a labeled streptavidin biotin method with a coupled HRP-peroxidase (LSAB2-Dako) was employed. After 3,3' diaminobenzidine tetrahydrochloride (DAB) staining, Harris hematoxylin was used for a slight counterstaining. The positive control for XIAP and Survivin expression was a normal stomach mucosa, and for p53, Bcl-2, and Ki-67 expression, previously determined positive tumor samples were utilized. As a negative control, the primary antibody was omitted. When more than one sample was available for a given patient, immunohistochemistry was performed in both the samples to evaluate possible discrepancies between samples from the same tumor.

Immunostaining results were analyzed by two independent observers and registered in an Eclipse E200 Nikon microscope connected to a Digital Sight System. For p53, Bcl-2, XIAP and Survivin, cases with fewer than 5% of positive tumor cells were considered negative, and cases with 5% or more positive tumor cells were considered positive. For ki-67 antigen, the cutoff of positivity was defined as 60% or more of positive tumor cells. Scoring analysis was performed in at least 10 fields in a 40 × magnification. Subcellular localization of XIAP and Survivin was evaluated as nuclear and/or cytoplasmic in all positive samples.

Statistical analysis

Statistical analysis was performed in the SPSS 17.0 software. PFS was evaluated as the time between the diagnosis and the progression of the disease. The events regarding PFS were considered progression of the disease (for the nonresponsive patients), relapse (for patients who achieved complete remission), or disease-related death. The remaining

cases were censored at the last follow-up. OS was evaluated as the time between the diagnosis and the end of the study. The event regarding OS was considered as disease-related death. The remaining cases were censored at the last follow-up. Co-expression of the proteins was evaluated through the Pearson χ^2 test with continuity correction when appropriated. Survival curves were plotted by the Kaplan–Meier method. The correlation between treatment response and proteins expression was examined by the log-rank test. For a 95% confidence interval, the difference between the analyzed groups was considered significant when $p < .05$.

RESULTS

Clinical, demographic, and treatment response data of centroblastic DLBCL patients

Median age at diagnosis was 58 years (range: 23–85 years). From the 81 patients included in this study, 12 were not treated with anthracycline-based protocols and were therefore excluded from the treatment response analysis. The remaining 69 patients were treated with anthracycline-based regimens and were therefore eligible for entering the prognostic analysis. PFS and OS ranged from 0 to 166 months with the median period being 4 months for PFS and 76 months for OS. Although PFS of the centroblastic DLBCL patients did not significantly differ from immunoblastic DLBCL patients, centroblastic DLBCL patients had better OS than immunoblastic DLBCL patients (Supplementary Figure 1). From the clinical and demographic features analyzed, the only characteristic that had an impact on PFS was age, with patients older than 60 years having a worse PFS than those younger than 60 years ($p = .002$). Although it was not significant, patients with low IPI tended to have a better PFS ($p = .098$). Regarding OS, low IPI ($p = .042$), and normal LDH level (.046) were favorable prognostic factors. A summary of patients' characteristics is listed in Table 1.

Apoptosis resistance related proteins expression in centroblastic DLBCL

For p53, Bcl-2, Ki-67, XIAP, and Survivin expression analysis, it was not possible to access p53 expression in 4 cases, Bcl-2 expression in 6 cases, Ki-67 in 15 cases, and XIAP expression in 10 cases. p53 expression was observed in 14 cases (18.2%), Bcl-2 expression was observed in 10 cases (13.3%), Ki-67 was observed in 41 cases (62.1%), XIAP expression was observed in 23 cases (32.4%), and Survivin expression was observed in 19 cases (23.5%). In Table 2, the positivity frequencies found in centroblastic DLBCL cases in our study, in contrast to the findings in DLBCL cases from other studies, are shown.

Another feature shown in Table 2 is the subcellular localization of the analyzed proteins. In this regard, the expression of p53 and Ki-67 antigen was detected in the nuclei of tumor cells and the expression of Bcl-2 was observed in the cytoplasm of tumor cells in all positive cases. With regard to the expression of XIAP, it was observed almost always in the nuclei of tumor cells (Figure 1(a)), whereas the expression of Survivin was detected almost always in the cytoplasm of tumor cells (Figure 1(b)) in all positive cases. In the XIAP positive cases, we observed that the positive cells usually had a larger and undifferentiated morphology than the negative ones even in the same tissue (Figure 1(a)).

Apoptosis resistance related proteins co-expression in centroblastic DLBCL

As these proteins have close relations in normal cell biology—e.g., p53 can inhibit the expression of Bcl-2 (20–23), XIAP (24, 25), and Survivin (26, 27), and has a role in cell cycle control (22, 28), whereas Ki-67 is also found throughout the cell cycle in cycling cells (29–31)—we investigated whether p53, Bcl-2, Ki-67, XIAP, and Survivin expressions were associated with one another in centroblastic DLBCL. However, although there was a trend for XIAP and Survivin expressions to have a direct correlation ($p = .059$), there was no significant correlation between the expression of any of the analyzed proteins ($p > .05$) (data not shown).

Proteins expression and treatment outcome of centroblastic DLBCL patients

As p53, Bcl-2, Ki-67, XIAP, and Survivin expressions have been correlated to a worse survival for DLBCL patients; we tested whether this was true for the centroblastic DLBCL subset as well. However, there was no correlation between p53, Bcl-2, Ki-67, and XIAP expression and PFS ($p = .968$, $p = .941$, $p = .737$, and $p = .742$, respectively) or OS ($p = .494$, $p = .939$, $p = .816$, and $p = .936$, respectively) (Table 1 and Figure 2).

On the other hand, there was a trend for Survivin expression to confer a poor PFS ($p = .077$) and OS ($p = .054$). Indeed, this tendency persisted when we performed the Mann–Whitney test to evaluate the correlation between the PFS of the Survivin-positive *versus* the Survivin-negative patients group (Table 3) ($p = .087$). In fact, in the Mann–Whitney test, Survivin expression correlated to a negative OS ($p = .045$). In particular, the Mann–Whitney test also corroborated the log-rank results for p53, Bcl-2, Ki-67, and XIAP expression (Table 3).

DISCUSSION

DLBCL is an aggressive form of NHL and exhibits a great heterogeneity in terms of molecular features and treatment response (1, 14, 32, 33). In the past decade, a great improvement has taken place in the treatment of DLBCL patients with the inclusion of the antibody, anti-CD20, in the treatment (rituximab) (34–36). However, it is a very expensive therapy and currently not all public health care services are able to include rituximab in their standard NHL treatment protocols. Anthracycline-based therapy is the most common treatment protocol, irrespective of whether it includes rituximab or not. Overall, it provides as much as 50% cure rate for DLBCL patients (1, 16, 17). In terms of morphology, this rate is higher for centroblastic DLBCL and lower for immunoblastic DLBCL (2–4), and represents distinct variants among the DLBCL. Although the morphology is often a subjective classification with the risk of variance

Table 1. Clinical and Demographic Data of the Centroblastic DLBCL Patients

Feature	All Included Patients – Absolute and Relative Frequencies (%)	Patients Included in the Prognosis Analysis – Absolute and Relative Frequencies (%)	PFS ± SE (%)	*p*	OS ± SE (%)	*p*
Total	81 (100)	69 (100)	22.6 ± 5.7		41.4 ± 8.3	
Age						
≤ 60 years	44 (54.3)	38 (55.1)	32.4 ± 8.8	0.002*	53.0 ± 9.6	.115
>60 years	37 (45.7)	31 (44.9)	9.1 ± 5.8		20.1 ± 15.0	
Gender						
Male	45 (55.6)	39 (56.5)	24.6 ± 7.3	0.950	47.3 ± 9.0	.897
Female	36 (44.4)	30 (43.5)	19.1 ± 9.9		25.7 ± 19.0	
Ethnicity						
White	58 (71.6)	51 (73.9)	22.7 ± 6.8	0.911	46.5 ± 9,8	.319
Non-white	23 (28.4)	18 (26.1)	24.3 ± 11.0		34.6 ± 13.6	
Tumor localization						
Nodal	67 (82.7)	58 (84.1)	19.4 ± 5.8	0.264	38.0 ± 8.9	.404
Extranodal	14 (17.2)	11 (15.9)	43.6 ± 15.5		61.4 ± 15.3	
Ann Arbor Stage						
Stage 1	22 (27.2)	17 (24.6)	25.7 ± 11.5	0.435	47.0 ± 13.3	.302
Stage 2	27 (33.3)	25 (36.2)	29.5 ± 11.3		30.1 ± 21.9	
Stage 3	16 (19.8)	15 (21.8)	17.8 ± 11.1		25.0 ± 14.2	
Stage 4	16 (19.8)	12 (17.4)	8.3 ± 8.0		57.1 ± 14.6	
International Prognostic Index						
Low (low and low-intermediate)	44 (54.3)	39 (56.5)	29.9 ± 8.6	0.098	49.0 ± 11.4	.042*
High (high and high-intermediate)	37 (45.7)	30 (43.5)	12.4 ± 6.5		32.8 ± 10.9	
LDH level						
Normal	59 (72.8)	49 (71.0)	27.3 ± 7.2	0.130	49.3 ± 10.1	.046*
High	22 (27.2)	20 (29.0)	11.7 ± 7.6		24.3 ± 12.6	
p53 expression						
Negative	63 (77.8)	54 (81.8)	24.5 ± 6.4	0.968	42.3 ± 9.4	.494
Positive	14 (17.3)	12 (18.2)	27.8 ± 14.8		33.3 ± 15.2	
N.D.	4 (4.9)	*N.A.*				
Bcl-2 expression						
Negative	65 (80.2)	55 (85.9)	26.4 ± 6.4	0.941	37.9 ± 10.6	.939
Positive	10 (12.3)	9 (14.1)	22.2 ± 17.8		50.0 ± 18.6	
N.D.	6 (7.4)	*N.A.*				
ki67 expression						
Negative	25 (30.9)	21 (36.8)	31.4 ± 10.4	0.739	38.8 ± 14.4	.816
Positive	41 (50.6)	36 (63.2)	22.9 ± 8.6		53.5 ± 9.1	
N.D.	15 (18.5)	*N.A.*				
XIAP expression						
Negative	48 (59.3)	41 (68.3)	25.3 ± 7.6	0.742	52.5 ± 9.1	.936
Positive	23 (28.4)	19 (31.7)	28.2 ± 12.2		50.4 ± 15.1	
N.D.	10 (12.3)	*N.A.*				
Survivin expression						
Negative	62 (76.5)	51 (73.9)	28.0 ± 6.8	0.077	47.6 ± 9.4	.054
Positive	19 (23.5)	18 (26.1)	0 ± 10.3		29.2 ± 12.9	

PFS: Progression-free survival. OS: overall survival. SE: standard error. Associations were investigated in the Kaplan-Meier plot by the log-rank test. *$p < .05$ was considered significant. N.D.: not determined. N.A.: not applicable.

Table 2. Positivity Frequencies of p53, Bcl-2, ki-67, XIAP and Survivin Expression in Centroblastic DLBCL Found in the Present Study and Positivity Frequencies of These Proteins in DLBCL Found by Other Groups

			Positivity Frequecy			
	Subcellular Localization	Number of Positive Cases/Total Number of Analyzed Cases	Centroblastic DLBCL	DLBCL*	References	*n***
p53	Nucleus	14/77	18.2%	13–38%	(39, 41, 43, 44, 95)	55–372
Bcl-2	Cytoplasm	10/75	13.3%	26–61%	(39, 41, 43, 44, 95, 96)	39–372
ki67	Nucleus	41/66	62.1%	18–100%	(37, 43, 45, 95)	55–405
XIAP	Nucleus	23/71	32.4%	26–67%	(13, 70, 96)	38–73
Survivin	Cytoplasm	19/81	23.5%	52–82%	(48, 96–99)	27–222

*Expression frequencies in DLBCL cases as reported by other groups. **Number of cases analyses in the cited studies.

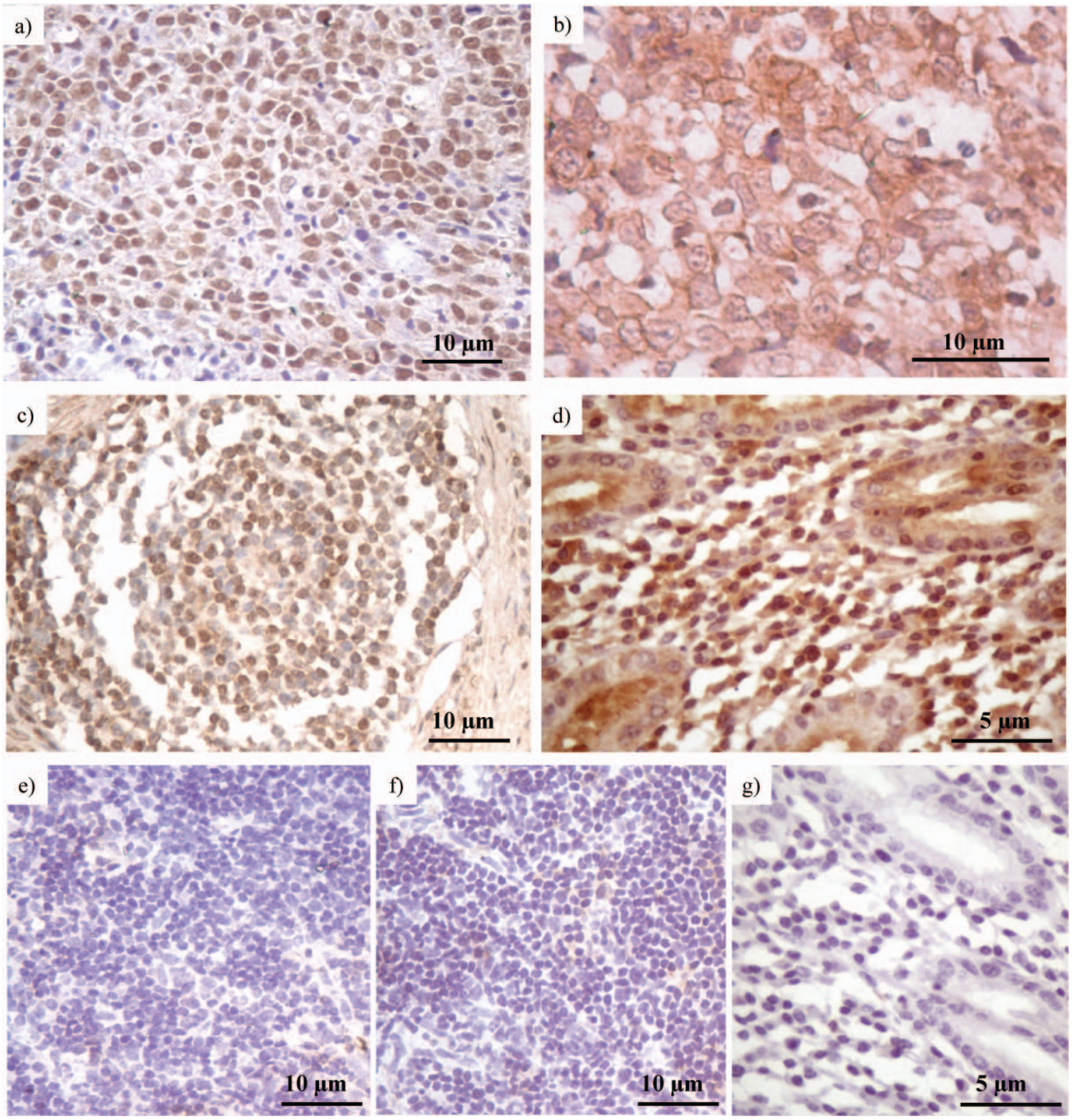

Figure 1. Imunohistochemical detection of XIAP and Survivin in centroblastic DLBCL cells. XIAP localized in the nucleus (a) while Survivin localized in the cytoplasm (b) of the tumoral cells. Positive expression controls were normal stomach mucosa tumor sections both for XIAP (c) and Survivin (d). Negative tumor cells for both XIAP (e) and Survivin (f) exhibit the same color pattern as negative control (omission of the primary antibody) (g).

among the observers, the fundamental basis of the classification criteria lies in the subtypes' greatest discrepancies: the centroblastic subtype is composed of large undifferentiated cells with modest cytoplasm and prominent nucleoli, while the immunoblastic subtype is composed of small rounded differentiated cells with little cytoplasm and dense nuclei (18, 32). More recently, a less subjective and, thus, more reliable classification foundation has become available as the immunoblastic subtype was shown to correlate with the non-GCB microarray/immunophenotypic profiling (11, 12). However, the centroblastic subtype has not been significantly correlated to the GCB microarray/immunophenotypic profiling, which left many questions regarding the issue of molecular alterations in the morphological subtypes unsolved.

Table 3. Mann-Whitney Test of Correlation Between p53, Bcl-2, ki-67, XIAP and Survivin Expression in Centroblastic DLBCL and Treatment Outcome (PFS and OS) of the Patients in the Study

	PFS		OS	
	Mann–Whitney U	*p*	Mann–Whitney U	*p*
p53	303.000	.712	295.500	.636
Bcl-2	241.000	.895	246.000	.977
ki-67	339.000	.502	288.000	.136
XIAP	389.000	.993	324.000	.298
Survivin	339.500	*.087*	312.500	.045*

Associations were investigated by the Mann-Whitney test. *$p < .05$ was considered significant. In italics, note the trend for Survivin expression to be associated with PFS.

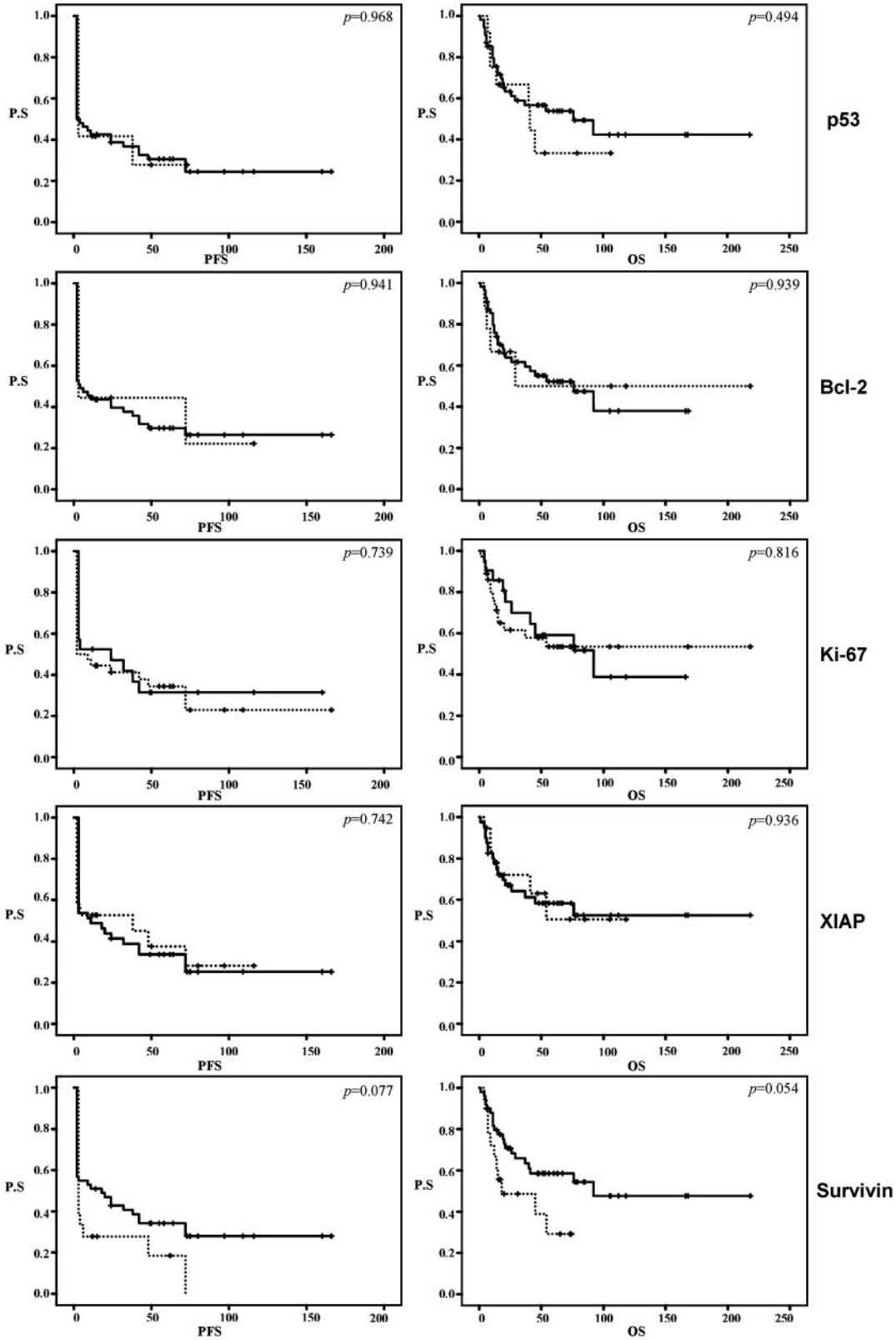

Figure 2. Kaplan–Meier survival curves showing the probability of survival (P.S.) in relation to the expression of the analyzed proteins. Progression-free survival (PFS) and overall survival (OS) of the centroblastic DLBCL patients do not seem to be affected by the expression of p53, Bcl-2, Ki-67, and XIAP. For each protein, straight curve represents the group of patients with negative tumor expression and dotted curve represents the group of patients with positive tumor expresion. Survivin was the only protein among the proposed prognostic factor that had borderline impact in the outcome of centroblastic DLBCL patients (bottom graphics).

In line with the differences between centroblastic and immunoblastic DLBCL in several aspects the expression of several apoptosis resistance related proteins has been analyzed in the present study, in a total of 81 patients with centroblastic DLBCL, to help elucidate the role of apoptosis resistance in the biology of centroblastic DLBCL. The analyzed proteins, e.g., p53, Ki-67, Bcl-2, XIAP, and Survivin, were selected based on having previously described roles in the pathobiology of DLBCL (13, 14, 37–48). Clinical and demographic characteristics of the centroblastic DLBCL patients did not differ from those observed in the whole group of DLBCL in the literature (1, 16, 17, 43, 49). Of note, treatment response of the centroblastic DLBCL patients was better than of the immunoblastic patients and corroborated the reported in the literature for both variants in the pre-Rituximab era (2–4).

Bcl-2, XIAP, and Survivin are important negative regulators of apoptosis. Irrespective of whether in the initial steps of the intrinsic pathway, through the modulation of cytochrome c release from the mitochondria (Bcl-2 family members), or in the final steps, through the inhibition of caspase activity (IAP family members), these proteins play a crucial physiological role, avoiding cell death due to minor and/or overcomable damage (50–53). p53 is one of the most important cell proteins being involved in the majority of cell processes, especially the cell cycle and apoptosis (21, 23, 28, 54–56). The ki-67 antigen is intimately related to the mitosis and, hence, its expression has been widely employed as a molecular tool to determine cell proliferation indices of tumor and healthy cell populations (29–31, 57–61). In association with other factors or in isolation, the expression of these proteins has been associated with the pathogenesis and treatment response of several tumor types, including DLBCL (17, 37, 43, 45–47, 62–69).

With regard to the expression rate of p53, Bcl-2, Ki-67, XIAP, and Survivin in centroblastic DLBCL, we observed that Bcl-2 and Survivin are present in a smaller frequency in centroblastic DLBCL than in the whole group of DLBCL, as shown in Table 2. This finding is in accordance with the fact that the expression of antiapoptotic proteins is not a common feature of centroblasts. Instead, they are common in immunoblasts (5, 6). It is indeed possible that as we selected off the cases of immunoblastic DLBCL, the expression frequencies of proteins that are more commonly expressed in their normal counterpart diminished.

On the other hand, XIAP is also known as a potent antiapoptotic protein, but its expression in centroblastic DLBCL is not inferior to that found for the whole group of DLBCL in the literature (13, 70). However, if we look at the IAPs subcellular localization, which is defining for their antiapoptotic function, Survivin does localize in the cytoplasm of the tumor cells, where it plays its antiapoptotic function, while XIAP does not localize in the cytoplasm as Survivin. Instead, XIAP expression has been found exclusively in the nuclei of tumor cells, which has been observed in DLBCL as well as in other cell models (13, 71–73), modulating its antiapoptotic function (65, 74). Nuclear translocation of XIAP has been observed by Russell *et al*. (74) and Nowak *et al*. (65) who identified hypoxia (74) and drug exposure (65) as stimuli involved in this phenomenon in their models. Therefore, in the case of DLBCL, XIAP does not appear to be present as an apoptosis counteractive protein, which may explain why its expression frequency did not vary in accordance with the morphological subtypes.

When the expression of Bcl-2, p53, and ki-67 was correlated with the treatment response in DLBCL, controversial results were obtained (37–47, 75, 76). These results suggest a possible influence of other proteins and/or cellular features in the ultimate determination of the prognosis of DLBCL. In the case of DLBCL, in particular, a relevant factor that may have influenced these conflicting results is the different proportions of centroblastic, immunoblastic, and other DLBCL variants in the composition of the studied case groups. As centroblastic and immunoblastic DLBCL are characterized by different protein expression patterns, it is possible that the expression of these proteins is associated with one of the variants, as further supported by the differences in the analyzed proteins expression rates as discussed earlier, thus biasing their prognostic value quest.

Interestingly, we observed a strong trend of correlation between XIAP and Survivin expression, which might not have been significant due to the limited number of cases in the present study. However, XIAP was almost exclusively found in the nuclei of the centroblastic DLBCL cells, which prevents it from inhibiting apoptosis (as discussed earlier) while Survivin was found in the cytoplasm, where it plays the IAPs classical function of caspase inhibition (72, 77, 78). Yet, at this point, the mechanism that leads to XIAP and Survivin co-expression in different cell compartments in these cells is not clear.

On the other hand, we did not find correlation between the expressions of any other proteins analyzed, despite the known relationships between, e.g., p53 and the others (20–28). This may correspond to the complex dynamics of protein expression control, where p53 is not the only regulator of XIAP, Survivin, and Bcl-2 expression (20, 79, 80). Alternatively, this may also be and indicative of the impairment of normal protein expression control often found in cancer cells.

Both XIAP and Survivin inhibitions have been shown to enhance apoptosis in DLBCL cells (81, 82). However, in centroblastic DLBCL cells, it might be possible that XIAP inhibition (physiologically performed by XAF1, which translocates XIAP to the nucleus, thus preventing its caspase-inhibiting activity (83)) is not essential for the cellular fate in terms of apoptosis inhibition or is impaired. Apoptosis inhibition by Survivin appears to be more important than that by XIAP in this model.

Survivin is an IAP family member that can act both in caspase inhibition when in the cytoplasm, and in mitosis appropriate progression when in the nucleus (84–86). Not only its expression, but also its subcellular localization, has been shown to predict prognosis for several types of tumors (68, 87, 88)). Survivin is widely expressed during fetal development, but is virtually absent in specialized differentiated tissues (78), which makes it a promising therapeutic target. In fact, small-molecule Survivin inhibitors are currently being

tested and the first clinical trials have demonstrated its safety (89, 90). Currently, Survivin inhibitors are being evaluated for DLBCL treatment (91).

Furthermore, there might be an apoptosis-prone pathway activated in these cells, leading to XIAP nuclear translocation, but a more crucial survival pathway might be surrogating the apoptotic signaling and maintaining survival through cytoplasmic Survivin antiapoptotic function. Indeed, in the centroblastic DLBCL patients analyzed, Survivin expression had a strong tendency of being a poor prognostic factor both for PFS and OS, as observed by both log-rank and Mann–Whitney tests. However, XIAP expression was not a PFS or OS prognostic factor in either tests, which reinforces the concept that nuclear XIAP does not exhibit an antiapoptotic function, and thus, does not stand as a determinant of the cellular fate and, ultimately, of the chemotherapy outcome.

On the other hand, the proposed role for cytoplasmic Survivin concerning apoptosis inhibition is a "passive" function as an XIAP ligand that stabilizes XIAP against different degradation stimuli, thus allowing XIAP to directly inhibit caspase activity (92, 93). In fact, the proapoptotic XIAP-associated factor 1 (XAF1)/XIAP complex has been shown to enhance Survivin degradation (94). However, in centroblastic DLBCL, XIAP nuclear localization, although may be mediated by XAF1, does not support this model; instead, these results support a more "active" role in which Survivin is able to inhibit apoptosis in the absence of XIAP in the cytoplasm.

In conclusion, taken together, our results point Survivin as an interesting treatment target for centroblastic DLBCL. Moreover, subcellular localization analysis of XIAP and Survivin highlights the importance of this feature for an IAP to exhibit its antiapoptotic role. Last, the differential expression of Bcl-2 and Survivin and the lack of prognostic value of p53, ki-67, Bcl-2, and XIAP in centroblastic DLBCL strongly support that DLBCL should be regarded as a group of different lymphomas with respect to molecular, morphological, and prognostic features. Therefore, each entity should be studied and treated considering its specificities.

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DECLARATION OF INTEREST

The authors declare no conflicting interests. The authors alone are responsible for the content and writing of the paper.

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