Title

Prognostic significance of survivin and caspase-3 immunohistochemical expression in patients with diffuse large B-cell lymphoma treated with rituximab and CHOP

Running title

Survivin and caspase-3 expression in DLBCL

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Abstract

Survivin is an inhibitor of apoptosis whose expression may be associated with inferior outcome in patients with diffuse large B-cell lymphoma (DLBCL) treated without rituximab. Caspase-3 is the final caspase of the apoptotic cascade and its pattern of expression may also be related to patients' outcome. In this study we investigated immunohistochemical expression of survivin and caspase-3 (CPP32) in 57 patients with DLBCL treated with rituximab and CHOP (R-CHOP). According to previously published criteria, we separately analyzed correlation of different types of survivin expression with patients' outcome. Nuclear survivin was expressed in only 26% of cases, cytoplasmic survivin was expressed in 81% of cases while application of immunoreactivity scoring system yielded 58% of survivin positive cases. Caspase-3 was expressed in 77% of cases. There were no significant correlations between any type of survivin expression and response to treatment or survival of the patients. The expression of caspase-3 was also not associated with patients' outcome. We conclude that survivin and caspase-3 have no significant prognostic significance in patients with DLBCL treated with R-CHOP.

Keywords non-Hodgkin's lymphoma, diffuse large B-cell lymphoma, survivin, caspase-3, prognostic factors, rituximab, immunohistochemistry

Abbreviations NHL – non-Hodgkin's lymphoma; DLBCL – diffuse large B-cell lymphoma; PS – performance status; ECOG – Eastern Oncology Cooperative Group; IPI – International prognostic index, LDH – lactate dehydrogenase; R-CHOP – rituximab, cyclophosphamide, doxorubicine, vincristin, prednisone; IAP – inhibitor of apoptosis, IRS –immunoreactivity scoring system, (u)CR – (unconfirmed) complete remission.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma (NHL) comprising 30-40% of all cases. Recently, rituximab in combination with CHOP (R-CHOP) was established as a new standard of care in patients with DLBCL [1,2]. Rituximab is an anti-CD20 monoclonal antibody with various mechanisms of action: antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), direct induction of apoptosis and increased chemosensitisation i.e. increased sensitivity to chemotherapy induced apoptosis [3]. Due to relevance of apoptosis for rituximab antitumor activity, the prognostic significance of immunohistochemical expression of survivin and caspase-3 was investigated in the present study. For this reason, the expression of bcl-2 was also evaluated.

Survivin belongs to inhibitors of apoptosis proteins (IAP) family. Although IAPs were originally described as caspase inhibitors, more recent studies identified survivin as a regulator of mitosis, a broad cytoprotective factor, and an effector of cellular adaptation to stress [4]. Survivin expression was detected in various tumors and mostly correlated with inferior outcome [5]. Immunohistochemical expression of survivin was also investigated in DLBCL [6-13]. However, the criteria of immunohistochemical staining positivity were rather inconsistent. The majority of studies evaluated nuclear positivity [6-9], two studies evaluated cytoplasmic positivity of survivin [10,11], a single study evaluated only intensity of staining [12] while in one study the positivity criteria were not specified [13]. Cut-off values were 30% percent [6,13], 10% percent [11] or IRS (immunoreactivity scoring system) was applied [7-9,12]. Eventually, survivin expression was associated with adverse outcome in four studies [6,9,10,12], while other four studies failed to demonstrate this relationship [7,8,11,13].

In contrast to survivin, few studies investigated the role of caspase-3 expression in lymphoma. Caspase-3 is the final caspase of the apoptotic cascade which activates cytolytic enzymes responsible for apoptosis. It was initially discovered that lymphoid tumors show stronger expression of caspase-3 than lymphoid hyperplasia [14]. Subsequently, Donoghue et al. demonstrated that immunohistochemical localization of caspase-3 can be correlated with clinical outcome in DLBCL [15]. More recent studies evaluated expression of active (cleaved) caspase-3 [16,17]. However, the expression of active caspase-3 is generally very low and it may be difficult to determine if active caspase-3 positive cells are indeed neoplastic.

Although prognostic significance of survivin and caspase-3 have been investigated in several studies, none of these studies included patients who were treated with rituximab containing regimens. We therefore investigated the expression of survivin and caspase-3 in our patients who were treated with R-CHOP.

Materials and Methods

This study included 57 consecutive patients with newly diagnosed DLBCL treated with R-CHOP in our institutions between 2003 and 2007. Inclusion criteria were available diagnostic paraffin block and planned treatment with 4-8 cycles of R-CHOP. Patients with HIV-associated lymphoma, primary central nervous system lymphoma or transformed lymphoma were excluded. Response to treatment was assessed according to standard criteria [18]. Complete remission (CR) and unconfirmed complete remission (uCR) after treatment with 4-8 cycles of R-CHOP +/- radiotherapy were considered as favorable response. Patients achieving partial remission, refractory or relapsed patients were treated with second line chemotherapy regimens including peripheral blood stem cell transplantation. Clinical parameters such as Ann-Arbor stage, performance status (PS) according to Eastern Cooperative Oncology Group (ECOG), extranodal involvement, age over or less than 60 and lactate dehydrogenase level (LDH) were recorded for each patient to calculate the International Prognostic Index (IPI) [19].

Standard sections for routine hematoxylin and eosin staining were obtained from each specimen to confirm the presence of lymphoma. All diagnostic biopsy specimens were reviewed by two hematopathologists. Immunohistochemical stains for CD20 (clone L26; dilution 1:200; DAKO, Glostrup, Denmark), CD3 (clone PC3/188A; dilution 1:50; DAKO, Glostrup, Denmark), survivin (lot DYX06, dilution 1:300, R&D Systems, Minneapolis, USA) caspase-3 (CPP32; clone JHM62, dilution 1:25, Abcam, Cambridge, UK) and bcl-2 (clone 3.1, dilution 1:50, Novocastra, Newcastle, UK) were performed using the streptavidin-biotin complex method. Nuclear and cytoplasmic expression of survivin was evaluated separately and the result was considered positive if $\geq$ 30% tumor cells were stained. The same cut-off value was used for bcl-2. In addition, survivin expression was assessed by IRS [8]. Intensity

of staining was designated as negative (0), weak (1), moderate (2) or strong (3). The percentage of survivin positive cells was scored as either no cells (0), less then 10% of cells (1), 10 – 50% of cells (2), 51 – 80% of cells (3) or over 80% of cells stained (4). By multiplication of these two parameters, IRS was calculated for each case. Cases were grouped as antigen negative (IRS 0 – 6) or antigen positive (IRS 7 – 12). Immunostaining for caspase-3 was considered positive if ≥ 50% tumor cells were stained [15].

The analyzed outcomes were response to treatment and overall survival (OS). Pretreatment features and response were compared using the $\chi 2$ test. OS curves were calculated according to the Kaplan-Meier method and the log-rank test was used for comparison between groups. Data were analyzed by Statistica software, version 8.0 (StatSoft. Inc., Tulsa, OK). A *P* value <0.05 was considered statistically significant.

The study was approved by the Ethics committees of the University Hospital Center Zagreb and School of Medicine, University of Zagreb.

Results

Patients' demographic and clinical characteristics with their response to treatment and survival are shown in Table 1. Out of 57 patients, 46 (81%) achieved complete or unconfirmed complete remission and 3-year OS was 66%. After a median follow up of 39 months, 10 patients who achieved complete remission relapsed and 19 patients died.

Nuclear survivin was positive in only 15 (26%) of patients (Fig. 1). In contrast, 46 (81%) showed cytoplasmic expression of survivin (Fig. 2). Both nuclear and cytoplasmic positivity was detected in 11 (19%) patients. When survivin was evaluated according to IRS, 33 (58%) cases were scored as positive (IRS 7-12). Caspase-3 was detected in all cases while 44 (77%) showed caspase-3 expression in more than 50% of tumor cells. Caspase-3 staining was diffuse cytoplasmic, with significant background from non-neoplastic cells (Fig. 3). We were not able to differentiate previously described punctuate and cytosolic patterns of caspase-3 staining [15]. Bcl-2 was positive in 45 (79%) samples.

Cytoplasmic expression of survivin correlated with bcl-2 expression (P=0.042). There were no significant correlations between any type of survivin expression, caspase-3 and bcl-2 expression with response to treatment (Table 2). Consecutively, there was no difference in OS according to the examined immunohistochemical parameters. However, patients with cytoplasmic expression of survivin had a trend toward inferior survival (P=0.072).

When patients were stratified in two prognostic groups according to IPI, there were 31 (54%) patients in the favorable (IPI 0-2) and 26 (46%) in the unfavorable group (IPI 3-5). In contrast to the immunohistochemical parameters, IPI was a strong predictor of response (94% for IPI 0-2 vs. 65% IPI 3-5; P=0.009) and survival (3y-OS was 83% for IPI 0-2 vs. 46% for IPI 3-5; P=0.001).

Discussion

Our results show that survivin and caspase-3 expression are not of prognostic significance in DLBCL treated with R-CHOP. Previously published studies on survivin in DLBCL had inconsistent results that supported or negated its adverse prognostic significance [6-13]. In our opinion, the principal reason for this discrepancy were inconsistent criteria of survivin positivity between studies. Therefore, we used three previously described criteria to evaluate results of immunohistochemical staining. We were unable to demonstrate a relation between survivin expression and outcome of patients by using any of these criteria. Nonetheless, cytoplasmic expression of survivin in comparison with nuclear expression showed a trend toward inferior response and survival, although not statistically significant. These results may in part correspond to the pivotal study of survivin in DLBCL by Adida et co-workers, who demonstrated an adverse role of cytoplasmic survivin on 222 patients [10]. Other reasons that may influence results are different clones of antisurvivin antibody used and differences in tumor tissue processing [5]. In contrast to all previous studies of survivin in DLBCL, our patients were treated with rituximab and CHOP. It is possible that rituximab can neutralize the proapoptotic role of survivin and, consequently, its adverse prognostic effect. Likewise, the addition of rituximab to CHOP overcomes the bcl-2 associated resistance to chemotherapy [20]. In vitro data to support a significant interaction between rituximab and survivin are very limited. Only a single experimental study showed that adding rituximab to an antisurvivin agent resulted in synergistic antitumor activity *in vitro* but not *in vivo*. [21] Recently, an inhibitor of survivin expression was tested in a phase I study and showed promising acitivity in patients with refractory lymphoma [22]. If antisurvivin agents are indeed effective in DLBCL, our data suggest that cytoplasmic expression of survivin should be used for treatment optimization.

Lack of prognostic significance of caspase-3 expression in our study could also be explained by the effect of rituximab. Nevertheless, more likely explanation is that caspase-3 cannot be evaluated properly because all tumor cells, as well as non-tumor cells, express this form of caspase-3. To avoid this problem, more recent studies tested the expression of the active form of caspase-3 [16,17]. However, it was demonstrated that prognostic value of active caspase-3 is closely related to expression levels of other apoptosis-related proteins. Besides, due to very small number of active caspase-3 positive cells, this marker requires a double-staining procedure that is inappropriate for routine diagnostic purposes.

Unlike immunohistochemical parameters, survival of our patients was significantly different when they were grouped according to IPI, the only universally accepted prognostic factor in DLBCL [19]. Because of limited number of patients in this study, this observation is also important for validation of our patients' cohort.

Our results show that survivin and caspase-3 cannot predict outcome of patients with DLBCL treated with R-CHOP. Different criteria for survivin positivity were used but none yielded prognostic significance. We conclude that survivin and caspase-3 are not useful prognostic markers in DLBCL.

Acknowledgments

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This article is in memory of our pathologist Marin Nola (1964-2008) who started this study.

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Fig. 1

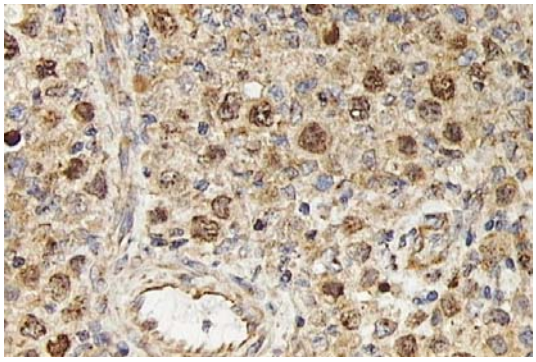

Fig. 2

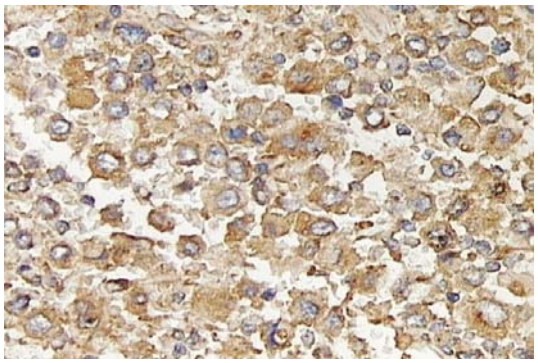

Fig. 3

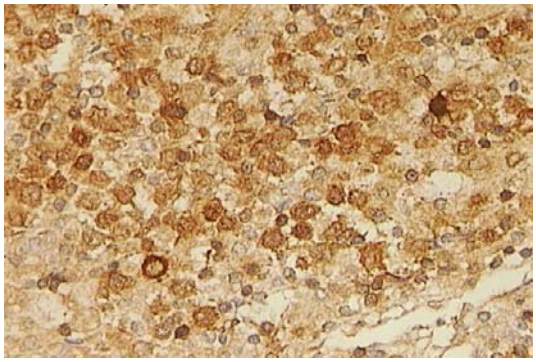

Figure legends:

Fig. 1 Strong nuclear staining for survivin. Original magnification x400.

Fig. 2 Cytoplasmic staining for survivin. Original magnification x400.

Fig. 3 Tumor cells show cytoplasmic expression of caspase-3. Original magnification x400.

Table 1 Patients' characteristics and response to the treatment

Characteristic	No. of patients (%) (N = 57)
Median age, range (years)	49 (17-75)
Male gender	33 (58)
Stage III or IV	32 (56)
Age ≥ 60 years	16 (28)
PS (ECOG) > 1	25 (44)
Elevated LDH	37 (65)
> 1 extranodal site	16 (28)
Response (CR+uCR)	46 (81)
3-year OS (%)	66%

PS performance status,
ECOG Eastern Oncology Cooperative Group,
LDH lactate dehydrogenase,
(u)CR (unconfirmed) complete remission

Table 2 Patients' outcomes according to immunohistochemical parameters and IPI

Characteristic	No. of patients (%)	Complete response (%)	*P*	3-year OS	*P*
Nuclear surviving					
positive	15 (26)	11 (73)	0.31	65%	0.90
negative	42 (74)	35 (83)		66%	
Cytoplasmic survivin					
positive	46 (81)	35 (76)	0.072	60%	0.072
negative	11 (19)	11 (100)		91%	
Survivin according to IRS					
0 – 6	24 (42)	22 (92)	0.071	59%	0.19
7 – 12	33 (58)	24 (73)		75%	
Caspase-3 expression					
positive	13 (23)	12 (92)	0.21	85%	0.13
negative	44 (77)	34 (77)		60%	
Bcl-2 expression					
positive	45 (79)	10 (22)	0.99	64%	0.48
negative	12 (21)	2 (17)		75%	
IPI					
0 – 2	31 (54)	29 (94)	0.009	83%	0.001
3 – 5	26 (46)	17 (65)		45%	

IRS immunoreactivity scoring system, IPI International prognostic index