Immunoexpression of Survivin in non-neoplastic lymphoid tissues and malignant lymphomas using a new monoclonal antibody reactive on paraffin sections

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Abstract Survivin is a member of the inhibitor of apoptosis gene family, which is also implicated in mitosis regulation. Most reports in the literature impute poor prognosis to neoplasms with overexpression of this protein. The purpose of the present study is to validate and compare the immunohistochemical reactivity of malignant lymphomas and reactive lymphoid tissue using a new mouse monoclonal antibody to Survivin produced in our laboratory, 6-78. Survivin was detected by immunohistochemistry on tissue microarrays. It was shown that the antibody anti-Survivin 6-78 reliably stains formalin-fixed, paraffin-embedded reactive and neoplastic lymphoid tissues, mostly in a nuclear

pattern. We confirmed using this novel antibody that Survivin immunostaining has a tendency to be lower in reactive lymphoid tissues and low-grade B cell lymphomas than in aggressive lymphomas. This antibody may represent a useful tool for standardizing the study of the immunoexpression of Survivin in neoplasms.

Keywords Malignant lymphomas · Survivin · Mouse monoclonal antibody 6-78 · Immunohistochemistry

Introduction

Survivin is a 16.5 kDa protein of 142 amino acids, representing the smallest member of the inhibitor of apoptosis family. The gene encoding Survivin is located on chromosome 17q25. This protein is associated with mitosis regulation, overexpressed in the G2/M phase and rapidly down-regulated after cell cycle arrest. Survivin also inhibits cell death via a mechanism that is still incompletely understood [1–8]. Survivin expression has been described mostly in fetal tissues and in neoplasms, while it is rarely expressed in adult tissues [1]. Survivin expression in human and mouse thymus has been reported, suggesting a role in T cell development [9, 10]. In active germinal centers in reactive lymph nodes, Survivin has been detected in the nuclei of blasts that were Bcl-2-negative [11].

The literature on the expression of Survivin in neoplasms is ever growing, and, in spite of some controversial data, the immunohistochemical detection of this protein is generally associated with tumor progression, recurrence, drug resistance, short survival rates, and poor prognosis [6].

Malignant lymphomas represent a heterogeneous group of neoplasms in which frequent overexpression of Survivin

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has been reported, mostly associated with worse prognostic factors [1, 16–28]. The series of lymphomas previously reported presents mostly one of their subtypes at a time and utilizes a polyclonal antibody to Survivin for immunohistochemical detection. In our laboratory a new monoclonal antibody (mAb) to a recombinant Survivin was recently developed, using the hybridoma technology. The purpose of the present study is to describe the immunoreactivity of different types of malignant lymphomas using this new anti-Survivin mAb. It was also intended to compare its expression in non-neoplastic lymphoid tissues to determine if this antibody could be of help in the differential diagnosis of benign and malignant lymphoid proliferations.

Material and methods

Production of anti-Survivin monoclonal antibody

BALB/c mice were immunized intraperitoneally four times with 100 µg of recombinant Survivin, at 2-week intervals in association with complete (first injection), incomplete (second and third injections), and without (fourth injection) Freund's adjuvant (EMD Biosciences, Inc, Calbiochem Brand, San Diego, CA, USA). Three days after the fourth immunization, fusion of the spleen cells with the non-Ig-producing myeloma cell line X63 Ag8 653 was performed using standard techniques [25]. When hybridoma growth could be detected, supernatants were tested for antibody-binding activity using an enzyme-linked immunosorbent assay. Subsequently, the reactivity of positive hybridomas was assessed by immunohistochemistry on frozen and paraffin-embedded tissue sections of some cases of non-Hodgkin's lymphomas (NHL) of B and T cell phenotypes. The 6-78 mAb was selected for further study because of its strong reactivity with some NHL cells, mainly nuclear, on frozen and paraffin-embedded tissue sections. The selected hybridoma was cloned by limiting dilution. Isotope characterization showed that 6-78 mAb belongs to an IgG1 subclass.

Immunoblotting analysis

Frozen tissue sections of seven cases of human NHL of B and T cell phenotypes were suspended in lysis buffer (20 mM Tris HCl pH 8, 150 mM NaCl, EDTA 4 mM, 0.5% Triton X-100, 0.2% SDS) containing a protease inhibitor mixture (protease inhibitor cocktail complete; Roche Diagnostics France S.A, Meylan, France) for 30 min, then sonicated briefly on ice. After centrifugation, the samples were separated by SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out in 12.5% gel and was followed by transfer of proteins to nitrocellulose mem-

branes. Nitrocellulose sheets were incubated in turn with 5% skimmed milk powder, to block free protein binding sites, and with 6-78 mAb. Bound antibody was targeted with goat anti-mouse peroxidase-conjugated immunoglobulins. The peroxidase activity was revealed using the enhanced chemiluminescence Western blotting detection system (Amersham, Little Chalfont, UK).

Immunostaining of various human normal and neoplastic tissues on paraffin sections

Different tissue microarrays (TMA) consisting of malignant lymphomas and non-malignant lymphoid tissues were tested in this study and are detailed in Table 1. All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. TMA sections were placed on superfrost plus slides, deparaffinized, and dehydrated for immunohistochemical technique. Antigen retrieval was performed in citrate buffer 10 mM, pH 6.0, two cycles ebullition of 10 min each; then, slides were allowed to cool at room temperature for 20 min; it followed by ebullition in EDTA buffer 1 mM, pH 8.0 for 5 min. Inhibition of endogenous biotin was performed using the kit provided by Dako (code X590, Dako S.A., Trappes, France). Incubation of sections with anti-Survivin monoclonal antibody 6-78 (undiluted supernatant) was performed for 30 min at room temperature. Revelation was achieved using StreptABComplex Duet reagent set (K0492, Dako, Carpinteria, CA, USA). As a nuclear and/or cytoplasm staining was expected, counterstaining was performed with light green. Positive controls consisted of known Survivin-positive NHL tissues, while omission of the primary antibodies in some sections served as negative control.

The labeling specificity was verified using competition experiments. The anti-Survivin mAb supernatants were pre-incubated for 30 min at room temperature with various dilutions of the recombinant Survivin, from 2 to 20 µg/ml, prior to immunodetection.

The reaction was evaluated in an optical microscope. Each spot of tissue was evaluated semiquantitatively as: 0=no positive cells; 1+=up to 25% positive cells; 2+=25–50% positive cells; 3+=50–75% positive cells, and 4+=more than 75% positive cells. Intensity of reaction was subjectively evaluated as: W=weak (score 1), M=moderate (score 2), or S=strong (score 3). Besides, reactivity was evaluated according to cell localization as N=nuclear, C=cytoplasm, or N+C=both.

In order to compare groups of lymphomas with non-neoplastic lymphoid tissues and among themselves, a final score (FS) was used, obtained by the addition of the score corresponding to the percentage of positive cells (0 to 4) and the score corresponding to the intensity of reactivity (0

Table 1 Survivin immunostaining of lymphomas and non-neoplastic lymphoid tissue with 6-78 monoclonal antibody

	Number of cases	NR	Percentage of positive cells				Intensity of immunostaining			Localization		
			1+	2+	3+	4+	W	M	I	N	NC	C
Non-neoplastic lymphoid tissue												
Lymph node	2	0				2		1	1	2		
Tonsil	5	1	1		2	1	2	1	1	4		
Spleen	6	5		1			1			1		
Thymus	5	2				3		3		3		
B cell lymphomas												
CLL	10	3				7	5	1	1	3	2	2
FOL	3	2				1	1			1		
HCL	1	1										
MCL	1					1		1		1		
SPLL	4	3				1			1	1		
DLCL-B	15	4				11	2	4	5	11		
DLCL-B, ALK ⁺	2	0			1	1	1	1		2		
T cell lymphomas												
Peripheral, NOS	20	5		1	4	10	2	5	8	14	1	
ALCL, ALK ⁺	64	22	2	4	5	31	16	19	7	38	5	1
ALCL, ALK ⁻	9	4			2	3	2	2	1	2	2	1
ALCL cell line	3	0			1	2	2	1				
Classical Hodgkin lymphoma												
CHL	15	10		3		2		3	2		3	2

Legends: *NR* non-reactive, 1+ up to 25% positive cells; 2+, 25–50% positive cells; 3+, 50–75% positive cells; and 4+, more than 75% positive cells; *W* weak, *M* moderate, or *S* strong, *N* nuclear, *C* cytoplasm, or *N+C* both nuclear and cytoplasm, *CLL* chronic lymphoid leukemia, *FOL* follicular lymphoma, *HCL* hairy cell leukemia, *MCL* mantle cell lymphoma, *SPLL* splenic marginal zone lymphoma, *DLCL-B* diffuse large B cell lymphoma, *NOS* not otherwise specified, *ALCL* anaplastic large cell lymphoma, *CHL* classical Hodgkin lymphoma

to 3). In this way, for each case, an FS varying from 0 to 7 was given.

Then, five groups were compared: (a) non-neoplastic lymphoid tissues, (b) low-grade B cell lymphomas, (c) diffuse large B cell lymphomas, (d) peripheral T cell lymphomas, not otherwise specified, (e) anaplastic large cell lymphoma either expressing or not ALK (anaplastic lymphoma kinase)-protein. To compare the FS of the five groups, the non-parametric Kruskal–Wallis test was used. To compare the FS for each group with the others, the non-parametric Mann–Whitney test was used. In addition, the Mann–Whitney test was also used to compare the FS of the 69 ALK-positive and the nine ALK-negative anaplastic large cell lymphomas (ALCLs).

Results

Immunoblotting analysis

SDS-PAGE analysis resulting in a single band of approximately 16 kDa was detected in five of the seven

NHL tissue samples with the 6-78 anti-Survivin mAb, consistent with the expected molecular weight of the Survivin protein confirming the specificity of this antibody (data not shown).

Immunoreactivity of 6-78 anti-Survivin mAb on paraffin-embedded tissue sections

Intensity and semiquantification of positive cells for each pathological process is described in Table 1 and illustrated in Figs. 1, 2, and 3. The present study was restricted to formalin-fixed tissues, as all preliminary tests performed using tissues fixed in Duboscq–Brasil (ethanol-based Bouin's fluid) resulted non-reactive (data not shown). Of note, the pre-incubation of the antibody with the recombinant Survivin led to a significant reduction of the signal intensity. A complete abolition of the reactivity was achieved at 20 µg/ml, confirming once more the specificity of this antibody.

In general, non-neoplastic lymphoid tissues showed positivity for Survivin in 10/18 cases (55%), being most often expressed in lymph nodes and tonsils but rarely in

Fig. 1 Expression of Survivin in non-neoplastic lymphoid tissue using 6-78 mAb: **a** moderate positivity in ca. 75% of lymphoid cells in tonsils; **b** weak positivity in less than 10% of cells in the spleen; **c**

strong positivity in ca. 30% of cells in the thymus; **d** strong positivity in virtually all lymphoid cells in a reactive lymph node (streptavidin–biotin–peroxidase; counterstain with *light green*; all figures $\times 400$)

the spleen. Among the low-grade B cell lymphomas, 10/19 cases were positive (52%). Eleven out of 15 diffuse large B cell lymphomas showed reactivity for Survivin (73%). The peripheral T cell lymphomas, not otherwise specified, expressed Survivin in 15/20 cases (75%), the anaplastic large cell lymphomas in 47/73 cases (64%), and the Hodgkin lymphomas in 5/15 cases (33%). Expression of Survivin was mostly present in the nuclei and in more than 50% of the tumor cells, and intensity was more frequently moderate to strong. In 2/7 positive cases of chronic lymphocytic leukemia, there was exclusive cytoplasmic expression. This was also observed in 2/73 anaplastic large cell lymphomas and in 2/5 positive Hodgkin lymphomas.

No statistically significant difference of the FS was shown among the five groups studied ($p=0.08$, Kruskal–Wallis test). When the FS of each group was compared with each other, no statistically significant differences were obtained (Table 2), nor when the FS of the 69 ALK-positive were compared with the nine ALK-negative ALCL ($p=0.58$, Mann–Whitney test).

Discussion

Detection of Survivin in pathological specimens of tumors seems to be clinically relevant, as it may be associated with worse prognostic features [7, 8]. The development of a monoclonal antibody brings advantages, as it allows production of unlimited amounts of antibody and higher standardization of immunohistochemical results, which is not always possible with polyclonal antibodies. Our results show that anti-Survivin antibody 6-78 reliably stains mainly the nucleus, and sometimes, the cytoplasm of reactive and neoplastic lymphoid cells. As small lymphocytes are inconsistently positive in reactive or neoplastic tissues, they are not suitable as internal positive controls of this marker. On the other hand, mitoses are consistently positive, in agreement with previous findings [26]. The present study is unique, since it compares the immunoexpression of Survivin in various types of lymphoma and non-neoplastic lymphoid tissue in the same setting, while introducing a novel antibody to this protein. Additionally, the predominant nuclear pattern

Fig. 2 Expression of Survivin in low-grade B cell lymphomas using 6-78 mAb (streptavidin–biotin–peroxidase; counterstain with *light green*): **a** weak positivity in ca. 80% of neoplastic cells in a follicular lymphoma ($\times 250$); **b** moderate positivity in virtually all cells in a

mantle cell lymphoma ($\times 400$); **c** strong nuclear positivity in virtually all cells in a chronic lymphoid leukemia ($\times 400$); **d** moderate cytoplasmic positivity in virtually all cells in a chronic lymphoid leukemia ($\times 1,000$)

of staining is corroborated, which has not always been emphasized in former studies.

The expression of Survivin in reactive lymphocytes is probably associated to the stage of development and activation of these cells as well as to their functional state. Among 18 non-neoplastic lymphoid tissues studied here, roughly the half showed expression of Survivin, in a proportion not statistically different from low-grade B cell lymphomas ($p=0.96$). Both groups tended to express Survivin in lower levels than large B cell lymphomas and peripheral T cell lymphomas, although this difference did not reach statistical significance ($p=0.06$ and 0.07). This finding limits the use of Survivin immunostaining for differential diagnosis with the low-grade B cell lymphomas, as previously proposed by Sugawara et al. [16]. In contrast to our findings on non-neoplastic lymphoid tissues, in a study on the expression of Survivin mRNA, Moriai et al. demonstrated that it was detectable in all 12 malignant hematopoietic cell lines and in 16 of 21 patients with hematologic malignancies, while it was undetected in normal leukocyte fractions [13]. In another study, cyto-

plasm expression of Survivin was frequently found in high-grade, but not in low-grade lymphomas, in peripheral blood leukocytes, lymph nodes, and spleen [1]. However, other authors have also found expression of Survivin in low-grade B cell NHL [11, 17, 19, 20, 23, 27].

In accordance with others, the large B cell lymphomas in the present set of cases frequently expressed Survivin (11/15 or 73%) [12, 14, 24, 28]. Likewise, our cases of peripheral T cell lymphomas were associated with frequent expression of Survivin (15/20 or 75%), which is in agreement with previous studies showing elevated levels of Survivin mRNA in these type of lymphomas [15, 22].

Forty-seven out of 73 (64%) ALCL expressed Survivin in the present study. This expression did not correlate with ALK status. In a study of 62 anaplastic large cell lymphomas (30 ALK-positive and 32 ALK-negative), Schlette et al. also found protein expression of Survivin in 34 cases (55%, of which 63% of the ALK⁺ cases and 47% of the ALK⁻ cases), without correlation with ALK status [21]. On the other hand, our results on Hodgkin lymphomas (only five positive cases out of 15 cases, 33%) are in

Fig. 3 Expression of Survivin in aggressive lymphomas using 6-78 mAb (streptavidin–biotin–peroxidase; counterstain with *light green*): **a** strong nuclear positivity in virtually all cells in a diffuse large B cell lymphoma ($\times 400$); **b** strong nuclear positivity in virtually all cells,

either medium or large, in a peripheral T cell lymphoma ($\times 400$); **c** strong positivity in virtually all cells in an anaplastic large cell lymphoma ($\times 320$); **d** another case of anaplastic large cell lymphoma lacking reactivity for Survivin, but mitotic figures are reactive ($\times 640$)

disparity with a study on 262 Hodgkin lymphomas, 234 of which showed nuclear positivity (89.3%) [18].

In conclusion, our results show that antibody anti-Survivin 6-78 reliably stains formalin-fixed, paraffin-embedded reactive and neoplastic lymphoid tissues, more frequently in a nuclear pattern. Expression of Survivin

occurs irregularly in reactive lymphoid cells and is mostly absent in the spleen. In general, Survivin presents a tendency to be less expressed in reactive lymphoid tissues and low-grade B cell lymphomas than in aggressive lymphomas. This antibody may represent a useful tool to standardize the reports on the expression of Survivin in neoplasms.

Table 2 Statistical analysis and comparative median and *p* values of Survivin immunostaining index of lymphomas and non-neoplastic lymphoid tissue with monoclonal antibody 6-78 (Mann–Whitney test)

	Number of cases	Median	Mean (standard deviation)	<i>p</i> values			
				B	C	D	E
A	18	3.0	2.88 (2.84)	0.96	0.07	0.07	0.51
B	19	5.0	2.94 (2.93)	–	0.06	0.07	0.48
C	15	6.0	4.60 (2.94)	–	–	0.89	0.07
D	20	5.5	4.50 (2.85)	–	–	–	0.08
E	73	5.0	3.42 (2.77)	–	–	–	–

Legends: *A* non-neoplastic lymphoid tissues, *B* low-grade B cell lymphomas, *C* diffuse large B cell lymphomas, *D* peripheral T cell lymphomas, not otherwise specified, *E* anaplastic large cell lymphoma with ($n=64$) and without ($n=9$) expression of ALK-protein

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Conflict of interests The authors do not declare any conflict of interests.

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