Anais da Academia Brasileira de Ciências (2013)
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201320120026
www.scielo.br/aabc

Evaluation of Bcl-2, Bcl-x and Cleaved Caspase-3 in Malignant Peripheral Nerve Sheath Tumors and Neurofibromas

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Manuscript received on December 11, 2012; accepted for publication on March 12, 2013

ABSTRACT

AIMS: To study the expression of Bcl-2, Bcl-x, as well the presence of cleaved caspase-3 in neurofibromas and malignant peripheral nerve sheath tumors. The expression of Bcl-2 and Bcl-x and the presence of cleaved caspase 3 were compared to clinicopathological features of malignant peripheral nerve sheath tumors and their impact on survival rates were also investigated. MATERIALS AND METHODS: The evaluation of Bcl-2, Bcl-x and cleaved caspase-3 was performed by immunohistochemistry using tissue microarrays in 28 malignant peripheral nerve sheath tumors and 38 neurofibromas. Immunoquantification was performed by computerized digital image analysis. CONCLUSIONS: Apoptosis is altered in neurofibromas and mainly in malignant peripheral nerve sheath tumors. High levels of cleaved caspase-3 are more common in tumors with more aggressive histological features and it is associated with lower disease free survival of patients with malignant peripheral nerve sheath tumors.

Key words: apoptosis regulatory proteins, malignant peripheral nervous sheath tumors, neurofibroma, Neurofibromatosis 1.

INTRODUCTION

Malignant peripheral nerve sheath tumors (MPNSTs) represent rare neoplasms, occurring in only 0.001% of general population (Ducatman et al. 1986). In individuals with Neurofibromatosis type 1 (NF1), they occur in 4.6% of all cases, being this syndrome the major risk factor for the development of MPNSTs (Ducatman et al. 1986).

MPNSTs may appear de novo or may develop from the transformation of a benign neural neoplasm, mainly from a plexiform neurofibroma, which occurs almost exclusively in NF1 (Scheithauer et al. 1999).

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Nevertheless, the molecular events involved in the development of neurofibromas and MPNSTs are still not completely understood. Abnormal programmed cell death is a hallmark of human tumors and apoptosis resistance is one of the most marking features of the majority of malignant neoplasms (Bray et al. 2009). Among the many known regulators and effectors of apoptosis, the family of caspases plays an important role in the execution phases of this type of cell death. Caspase-3 is considered to be the central protein in the execution of apoptosis and the detection of its activated form (cleaved caspase-3; CC3) is a valuable tool to detect apoptotic cells, even before the morphological features of apoptosis are present (Arai et al. 2005). Many proteins participate in the regulation of apoptosis; between them, there are the Bcl-2 family proteins, which include pro-apoptotic, e.g. Bax protein, and anti-apoptotic proteins, e.g. Bcl-2 and Bcl-x_L.

The altered expression of Bcl-2 proteins family members and CC3 has been analysed in various benign and malignant neoplasms and their prognostic value has been demonstrated (Arai et al. 2005, Friess et al. 1998, Satoh et al. 2000, Biroccio et al. 2001). Nevertheless, few studies were performed with neurofibromas and MPNSTs and, to our knowledge, there is no study that investigated the presence of Bcl-2, Bcl-x and CC3 in these tumors and their the correlation to the clinicopathological features of MPNSTs and their impact on survival rates.

Studies that investigated the Bcl-2 expression in MPNSTs present different results, showing that there is still a lack of understanding of the involvement of this protein in their pathogenesis (Suster et al. 1998, Hirakawa et al. 1996, Miettinen et al. 1998, Dan'ura et al. 2002, Thanakit et al. 2006, Köhler et al. 2002). These divergent results may be a consequence of the small number of cases included in many investigations and also of the fact that most studies investigated MPNSTs together with other sarcomas, probably reflecting the difficulty of conducting research with larger number of cases of MPNSTs due to its rarity. Soft tissue sarcomas represent a heterogeneous group of tumors with different clinical behaviors. Therefore, a question raised about the studies evaluating prognostic factors in various histological types of sarcomas taken together, is that although the findings may be applicable to many sarcomas, certainly they not apply to all types sarcomas. Another important factor is that all previous studies used conventional pathologist-based manual scoring to quantify Bcl-2-staining, which increases inter-observer and intra-observer variability.

The aim of this research was to study the expression of Bcl-2, Bcl-x, as well the presence of CC3 in MPNSTs and neurofibromas. The expression of Bcl-2 and Bcl-x and the presence of CC3 were compared to clinicopathological features of MPNSTs and their impact on survival rates were also investigated.

MATERIALS AND METHODS

Ethical Committee of National Institute of Cancer (INCA), RJ, Brazil, approved this study.

Sample

The study sample comprises 16 cases of plexiform neurofibromas, 22 cases of non-plexiform neurofibromas (from NF1-patients and from non-NF1 patients), and 28 cases of MPNSTs, which were obtained from the pathology files of INCA and Hospital Universitário Antônio Pedro of the Universidade Federal Fluminense. All the cases were used in previous studies (Cunha et al. 2003, 2008, 2009). The samples of the tumors were included in two tissue microarray (TMA) paraffin blocks. For detailed information about the construction method for TMA block, please refer to the previous published paper (Cunha et al. 2012)

All the cases were reviewed by two pathologists. For neurofibromas, only cases with heterogeneous and diffuse immunostaining for S-100 protein were included. The samples of MPNSTs included in this study were obtained from the paraffin blocks of

the resected primary tumor (in cases submitted to radiotherapy and/or chemotherapy prior to primary tumor resection, the biopsy material was used, since it had the same histological grade of the resection material). MPNSTs with one of the following features were included: arose within a peripheral nerve; arose in transition from a benign neural tumor; developed in a NF1 patient and exhibited the same histological features of most MPNSTs originating from a nerve; and developed in a non-NF1 patient, exhibited the same histological features as most MPNSTs and expressed S-100 protein (Dako Corp., Carpinteria, CA, USA, 1:4,000) and/or CD57 (clone TB01; Dako, 1:50). All samples were immunoreactive for anti-vimentin (clone V9; Dako, 1:800) and negative for anti-cytokeratin (clone AE1/ AE3; Dako, 1:400), anti-melanosome, (clone HMB-45, 1:200), anti-actin (smooth muscle; clone 1A4; Dako, 1:250), anti-actin/muscle (clone HHF35; Dako, 1:1,000), and anti-desmin (clone D33; Dako, 1:100) antibodies, except the malignant Triton tumors, which exhibited anti-actin/muscle and desmin immunopositive areas.

Clinical and Pathological Features of MPNSTs Included in the Study

Tables I and II show the clinical and pathological features of MPNSTs included in this research, respectively. MPNSTs were classified in low and high-grade (Scheithauer et al. 1999). Tumors were classified as low-grade if they presented mild to moderate cellularity, mild nuclear atypia, absence of necrosis and no or few mitoses (mitotic index <4). Tumors were classified as high-grade if they had marked cellularity, variable cellular pleomorphism and easily identified mitoses (mitotic index ≥ 4). Mitotic index was calculated by the sum of mitotic figures in 10 high-power fields. In each case, at least 30 fields were analyzed and the mean value of the sum of mitotic figures in each 10 high-power fields was obtained.

Epithelioid MPNSTs included in this study were composed predominantly of epithelioid cells and presented spindle cells identical to that of conventional MPNSTs. Malignant triton tumors had areas of cells with rabdomyoblast morphology, which expressed desmin and/or actin/muscle.

Immunohistochemical Staining

Sections with 3 μm from the two TMA paraffin blocks were cut and collected on silane-coated slides. After dewaxing, proteins were demonstrated by IHC, using the protocol described in a previous study (Cunha et al. 2009). Sections were incubated overnight at 4°C with a 1:200 dilution of the primary antibody anti-Bcl-2 (clone 124; Dako Corporation, USA), with a 1:25 dilution of the primary antibody anti-Bcl-x (clone A35-10; Dako Corporation, USA), and with a 1:600 dilution

TABLE I
Clinical data of patients with malignant peripheral nerve sheath tumors.

Patient	Age at diagnosis	Sex	Race	NF1	Site	Free surgical margins	Treatment	Local recurrence	Metastasis	Site of metastasis	Death	Overall survival (months)	Disease-free survival (months)
1	27	F	—	Yes	Lumbar	No	Resection	Yes	No		No	21.60	18.23
2	44	F	W	No	Left elbow	No	Resection + Rxt	No	No		No	13.60	13.60
3	29	F	—	Yes	Right flank	No	Resection + Rxt	No	No		Yes	12.47	12.47
4	23	F	B	Yes	Pelvis	No	Resection	No	No		Yes	4.87	4.87

—, information not avaiable; **F**, female; **M**, male; **B**, black; **W**, white; **Rxt**, radiotherapy; **Ct**, chemotherapy;
* these patients presented metastasis at the moment of the tumor diagnosis.

TABLE I (continuation)

Patient	Age at diagnosis	Sex	Race	NF1	Site	Free surgical margins	Treatment	Local recurrence	Metastasis	Site of metastasis	Death	Overall survival (months)	Disease-free survival (months)
5	28	F	W	Yes	Right flank	Yes	Resection	No	No		No	76.13	76.13
6	32	F	W	No	Left lower limb	Yes	Resection	No	Yes	Lung	No	12.10	.00*
7	34	F	W	No	Right lower limb	Yes	Resection	No	No		No	44.77	44.77
8	40	F	W	No	Breast	No	Resection + Rxt	No	No		No	96.90	96.90
9	20	F	—	Yes	Lumbar	—	Rxt	No	No		Yes	1.83	1.83
10	53	F	B	Yes	Left shoulder	Yes	Resection	No	No		Yes	10.87	10.87
11	21	F	W	Yes	Left lower limb	Yes	Resection	No	No		No	67.50	67.50
12	23	M	W	Yes	Left lower limb	—	Ct	No	Yes	Lung	Yes	2.73	.00*
13	42	F	W	No	Pelvis	No	Resection + Rxt	No	Yes	Liver and pancreas	Yes	11.30	9.60
14	68	M	W	No	Right upper limb	—	Resection + Rxt	Yes	No		No	9.50	7.00
15	19	M	W	No	Right upper limb	—	Rxt	No	Yes	Lung	No	16.10	.00*
16	23	F	W	Yes	Sacrum and spine	—	Rxt	No	Yes	Lung	Yes	7.73	7.67
17	34	F	B	Yes	Abdomen	No	Resection	Yes	No		No	10.10	5.43
18	60	F	B	Yes	Right lower limb	Yes	Resection	No	Yes	Lung	No	40.80	22.83
19	40	F	B	No	Thorax	Yes	Resection + Rxt	No	No		No	61.10	61.10
20	78	F	W	No	Face sinus	No	Resection + Rxt	Yes	No		Yes	77.90	50.20
21	24	M	W	Yes	Right upper limb	—	Biopsy + Ct	No	Yes	Lung	Yes	12.80	6.60
22	45	M	B	Yes	Abdomen	—	Resection	Yes	Yes	Lung	Yes	14.70	13.07
23	63	M	B	Yes	Supraclavicular	Yes	Resection	No	No		Yes	60.30	60.30
24	85	M	W	No	Head (temporal)	Yes	Resection	No	No		Yes	20.20	20.20
25	72	F	W	No	Left lower limb	Yes	Resection + Rxt	No	Yes	Lung	Yes	42.00	39.93
26	80	F	W	No	Right foot	Yes	Resection	Yes	No		Yes	13.40	8.10
27	30	F	B	No	Thorax	Yes	Resection + Rxt	No	Yes	Bone	No	35.70	4.67
28	41	M	B	Yes	Right lower limb	—	Rxt	No	Yes	Cervical region	Yes	7.20	6.97

—, information not avaiable; **F**, female; **M**, male; **B**, black; **W**, white; **Rxt**, radiotherapy; **Ct**, chemotherapy;
* these patients presented metastasis at the moment of the tumor diagnosis.

TABLE II
Pathological data of the malignant peripheral nerve sheath tumors.

Case number	Size (cm)	Grade	Presence of heterologous differentiation	Necrosis	Mitotic index (mitotic figures in 10 high-power fields)*
1	4.6	high	no	no	7.0 (sd=2.0)
2	17.0	high	rabdomiosarcomatous areas	yes	9.3 (sd=1.5)
3	15.0	high	no	yes	5.3 (sd=1.2)
4	21.0	high	rabdomiosarcomatous areas	yes	17.0 (sd=2.0)
5	7.0	low	chondrosarcomatous areas	no	3.7 (sd=1.5)
6	21.0	high	no	yes	7.0 (sd=3.5)
7	10.0	high	no	no	9.0 (sd=2.0)
8	16.0	low	no	no	0.3 (sd=0.6)
9	—	high	no	no	18.0 (sd= 4.0)
10	28.0	high	no	yes	19.0 (sd=1.0)
11	17.0	high	no	yes	13.0 (sd=2.6)
12	—	high	no	yes	10.7 (sd=1.5)
13	13.0	high	chondrosarcomatous areas	yes	10.3 (sd=5.7)
14	—	high	no	no	10.3 (sd=4.0)
15	—	low	epithelioid	no	3.0 (sd=0.0)
16	—	high	no	yes	7.0 (sd=2.6)
17	13.0	high	epithelioid	yes	7.3 (sd=1.2)
18	12.0	high	chondrosarcomatous area	no	6.0 (sd=2.0)
19	6.0	low	no	no	2.3 (sd=1.5)
20	—	low	no	no	3.0 (sd=1.0)
21	—	high	no	yes	7.0 (sd=2.0)
22	23.0	high	no	yes	13.3 (sd=3.2)
23	5.0	high	chondroma area	no	7.0 (sd=2.0)
24	10.0	low	no	no	3.7 (sd=1.5)
25	13.0	low	no	no	3.7 (sd=1.2)
26	3.0	high	no	no	6.7 (sd=0.6)
27	11.0	high	no	no	13.0 (sd=3.0)
28	—	high	no	yes	8.0 (sd=1.0)

—, information not available (the tumor resection was performed in other institution); sd, standard deviation
*, in each case, at least 30 fields were analyzed and the mean value of the sum of mitotic figures in each 10 high-power fields is showed in the table.

of the primary antibody anti-CC3 (clone Asp175; Cell Signaling Technology, USA). Palatine tonsil was used as a positive control. The negative control was established by omission of primary antibody.

The glass slides were reviewed independently by two pathologists and were scored as negative or positive. The location (nucleus, cytoplasm and/or cytoplasmic membrane) of immunopositivity

for Bcl-2, Bcl-x and CC3 was also evaluated. Following the independent evaluation of the immunohistochemical staining, if there were any divergence between the pathologists, the glass slides were discussed at a separate evaluation in order to reach a consensus on the evaluation. For the cases classified as positive, quantification of Bcl-2, Bcl-x and CC3 immunostaining was performed by computerized digital image analysis (Image-Pro Plus software v4.5; Media Cybernetics), as previously described (Cunha et al. 2009), and expressed as positivity index (PI), defined as positive area divided by total tissue area.

To avoid searching the optimal cut-off point for the immunostaining to perform the statistical analyzes (overall and disease-free survivals), which could be misleading and associated with difficulty in its interpretation, we arranged the cases of MPNSTs in ascending order according to PI values and divided them into two groups: one with tumors with high PI (cases in the third third of the list) and another with tumors with low PI (cases in first two-thirds of the list).

Statistical Analysis

Clinicopathological and immunohistochemical variables were compared by Chi-square, Fisher's exact, Student t, and Mann-Whitney tests. The Kaplan-Meier method was used to evaluate survival curves and statistical significance of clinicopathological variables was determined by log-rank test. Multivariate analysis was performed by Cox-regression model. SPSS software v.11 was used for statistical analysis. Differences were considered significant if $p<0.05$.

RESULTS

Data regarding the immunostaining for Bcl-2, Bcl-x and CC3 in neurofibromas and MPNSTs are described in Table III.

TABLE III
Immunohistochemical data of neurofibromas and of malignant peripheral nerve sheath tumors.

Neurofibromas							
Antibody	**Group**	**% positive cases (n)**	**Mean of PI**	**Standard Deviation**	**Mean of PI**	**IP min / max**	**IQ Range**
Anti-Bcl-2	**Neurofibromas**						
	Plexiform neurofibromas	18.8% (n=3)	0.0299	0.0266	0.0285	0.0000 / 0.0571	—
	Non-plexiform neurofibromas	42.9% (n=9)	0.0789	0.1081	0.0355	0.0000 / 0.3583	0.0570
Anti-Bcl-x	**Neurofibromas**	43.2% (n=16)	0.0043	0.0055	0.0019	0.0000 / 0.0171	0.0091
	Plexiform neurofibromas	37.5% (n=6)	0.0046	0.0050	0.0044	0.0000 / 0.0094	0.0092
	Non-plexiform neurofibromas	47.6% (n=10)	0.0041	0.0061	0.0019	0.0000 / 0.0171	0.0064
Anti-CC3	**Neurofibromas**	100% (n=28)	0.0044	0.0486	0.0275	0.0000 / 0.0240	0.0545
	Plexiform neurofibromas	100% (n=28)	0.0347	0.0038	0.0025	0.0000 / 0.0130	0.0036
	Non-plexiform neurofibromas	100% (n=28)	0.0050	0.0055	0.0027	0.0004 / 0.0240	0.0070
Malignant Peripheral Nerve Sheath Tumors (MPNSTs)							
Antibody	**Group**	**% of positive cases (n)**	**Mean of PI**	**Standard Deviation**	**Mean of PI**	**IP min / max**	**IQ Range**
Anti-Bcl-2	MPNST	78,6% (n=22)	0.4684	0.0543	0.2500	0.0000 / 0.2176	0.0724
Anti-Bcl-x	MPNST	75.% (n=21)	0.0347	0.0526	0.0054	0.0000 / 0.2036	0.0533
Anti-CC3	MPNST	100.0% (n=28)	0.1672	0.1352	0.0990	0.0324 / 0.5346	0.1900

CC3, cleaved caspase 3; PI, positive index; min, minimum; max, maximum; IQ, interquartile.

Cellular Location of Immunostaining for BCL-2, BCL-X and Cleaved Caspase-3

Immunostaining for proteins Bcl-2 and Bcl-x was found in the cytoplasm of tumor cells of neurofibromas and MPNSTs (Fig. 1). Moreover, expression of Bcl-2 and Bcl-x was also observed in the endothelial cells of blood vessels, as well as in lymphocytes, when present in the tumor. Regarding the presence of CC3, neurofibromas showed only nuclear immunostaining (Fig. 2A). In contrast, half of MPNSTs showed only nuclear immunostaining and the other 50% of the cases showed cytoplasmic and nuclear staining (Fig. 2B, 2C and 2D).

Comparison of Immunostaining for BCL-2, BCL-X and Cleaved Caspase-3 Between the Different Types of Neurofibromas

There was no statistically significant difference between plexiform neurofibromas and non-plexiform neurofibromas regarding the percentage of tumors that expressed Bcl-2 (Fisher's exact test, p=0.166) and Bcl-x (Chi-square test; p=0.539) proteins. With respect to CC3, all neurofibromas were immunopositive, expressing the protein only in the nucleus.

Considering the immunopositive cases for each antibody, there was no statistically significant difference of PIs between the two groups of

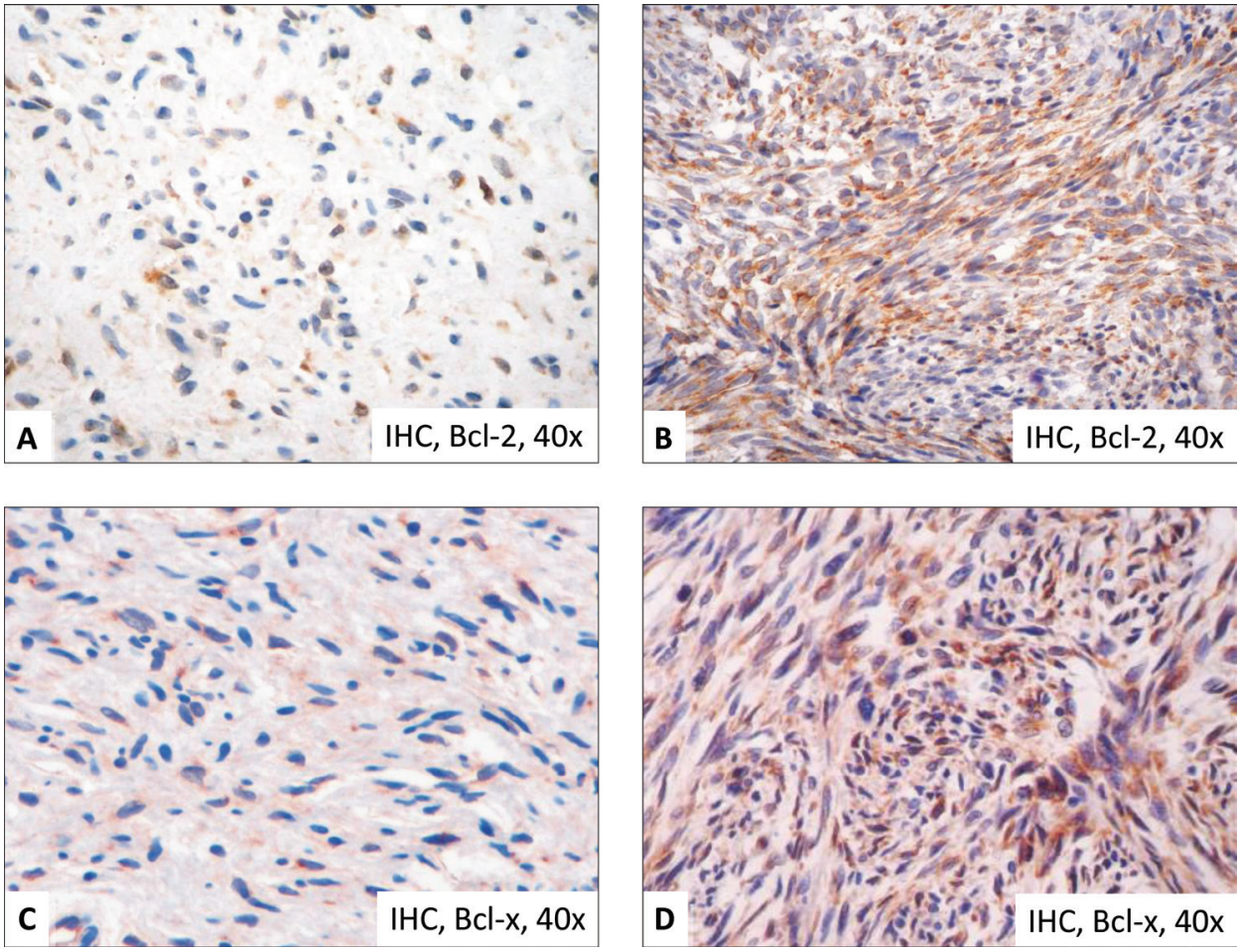

Fig. 1 - A. Neurofibroma showing cytoplasmic immunostaining for Bcl-2; **B**. MPNST with cytoplasmic immunostaining for Bcl-2; **C**. Neurofibroma showing cytoplasmic immunostaining for Bcl-x; **D**. MPNST with cytoplasmic immunostaining for Bcl-x.

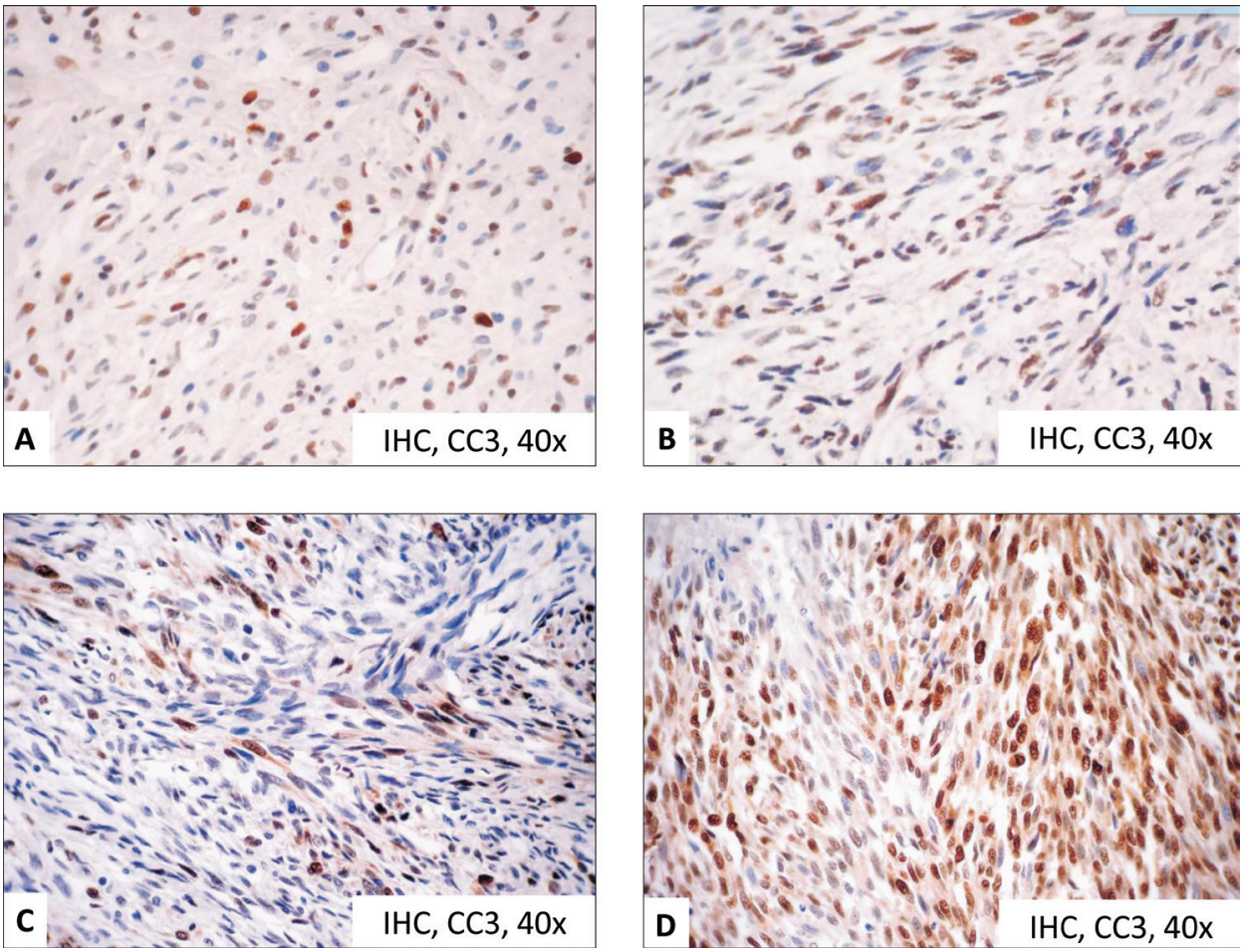

Fig. 2 - A. Neurofibroma showing nuclear immunostaining for CC3. **B.** MPNST showing nuclear immunostaining for CC3. **C and D.** MPNST showing nuclear and cytoplasmic immunostaining for CC3.

neurofibromas: Bcl-2 (Mann-Whitney test, p=0.373), Bcl-x (Mann-Whitney test, p=0.562) and CC3 (Mann-Whitney test, p=0.554).

Comparison of Immunostaining for BCL-2, BCL-X and Cleaved Caspase-3 Between Malignant Peripheral Nerve Sheath Tumors and Neurofibromas

There were more positive MPNSTs for Bcl-2 (Chi-square test, p <0.001) and Bcl-x (Chi-square test; p <0.0001) than neurofibromas. Bcl-2 PI in positive neurofibromas did not differ statistically from the PI of positive MPNSTs (Mann-Whitney test, p=0.989). In contrast, positive MPNSTs showed higher Bcl-x PI than positive neurofibromas (Mann-Whitney test, p=0.002). All neurofibromas and MPNSTs expressed CC3. Nevertheless, MPNSTs showed higher CC3 PI than neurofibromas (Mann-Whitney test, p<0.001).

Comparison of Immunostaining for BCL-2, BCL-X and CC3 with Clinicopathological Features of Cases of Malignant Peripheral Nerve Sheath Tumors

We investigated the correlation of the immunostaining of Bcl-2, Bcl-x and CC3 with the presence of NF1, recurrence and metastasis, histological grade, mitotic index and necrosis. In all tests performed, presence of NF1 (Fisher's exact test, p=0.011), high histological grade (Fisher's exact test, p=0.003) and high mitotic index (Fisher's

exact test, 0.006) were correlated to the presence of CC3 in the cytoplasm of MPNSTs cells. There was also statistically significant correlation between high CC3 PI in MPNSTs with high histological grade (Mann-Whitney test, p=0.012), high mitotic index (Mann-Whitney test, p=0.037) and necrosis (Mann-Whitney test, p=0.010)

Overall and Disease Free Survival and Prognostic Factors in Cases of Malignant Peripheral Nerve Sheath Tumors

The overall 5-year survival rate of patients with MPNST was 46%. Considering the patients who received curative treatment, the 5-year disease-free survival rate was 39%. All these data were showed in the previous study in which we used the same sample (Cunha et al. 2012). In that study, we evaluated the influence of clinical and pathological features on overall and disease free survival in patients with MPNSTs: sex, presence of NF1, tumor location, treatment performed, status of the surgical margins, local recurrence, metastasis, histological grade, mitotic index, necrosis, epithelioid variant, Triton tumor and chondrosarcoma differentiation. The variables, which were significant in univariate log-rank analysis for survival rates, are shown in Table IV, as well as the results of the evaluation of Bcl-2, Bcl-x and CC3. Presence of NF1 (p=0.044), high histological grade (p=0.045), presence of necrosis (p= 0.003) and high CC3 PI (p=0.024) were significant in univariate log-rank analysis for shorter overall survival and were included in Cox regression model, which showed that the presence of necrosis was an independent prognosis factor for lower overall survival (p=0.007). (Table V) Epithelioid variant (p=0.036) and high CC3 PI (p<0.001) were significant in univariate log-rank analysis for disease-free survival (Table II). Cox regression model showed that high CC3 PI could be considered an independent prognosis factor for disease-free survival (p=0.002) (Table V).

DISCUSSION

To our knowledge, the present study was the first to use computerized image analysis to calculate the Bcl-2 expression in neurofibromas and MPNSTs. The results showed a significantly higher percentage of positive MPNSTs for Bcl-2 than neurofibromas (p<0.001). Other studies also observed increased expression of Bcl-2 in malignant neoplasms, such as cutaneous carcinomas and melanomas, when compared to their benign counterparts, suggesting that the expression of Bcl-2 protein could be an oncogenic factor (Leiter et al. 2000, Hussein et al. 2004). Corroborating to our findings, other studies also observed that most MPNSTs express Bcl-2 (Miettinen et al. 1998, Watanabe et al. 2001, Sabah et al. 2007). Contrary to our results, Suster et al. (1998) found that 87.5% of eight neurofibromas expressed Bcl-2, while only 39.3% of 25 MPNSTs were immunoreactive. In another survey with 38 sarcomas, none of the four studied MPNSTs expressed the Bcl-2 protein. In two studies that included MPNSTs, which arose from neurofibromas (one study with eight and the other with 20 cases), there was no difference in Bcl-2 expression between the benign and malignant counterparts (Thanakit et al. 2006, Watanabe et al. 2001).

Bcl-2 expression has been correlated with histological grade and clinical behavior of several malignant neoplasms (Hirakawa et al. 1996, Sabah et al. 2007, Silvestrini et al. 1994, Ofner et al. 1995, Groeger et al. 2004). In certain cancers, like breast carcinomas, the expression of this protein is more commonly observed in low-grade neoplasms, whereas in other tumors, such as bladder carcinoma and some sarcomas, its expression has been more common in high-grade tumors (Sabah et al. 2007, Yang et al. 2000, Eissa and Seada 1998). It has been demonstrated that the immunoreactivity to the Bcl-2 is associated with increased survival in patients with colorectal, breast, lung and ovary carcinomas (Silvestrini et

TABLE IV
Results of the influence of variables on overall and disease-free survival in patients with malignant peripheral nerve sheath tumors.

Variables	*Total SV (months)*	*Total SV (p log-rank)*	*Disease-free SV (months)*	*Disease-free SV (p log-rank)*
NF1				
Yes	31.6	**0.044**	31.4	0.959
No	59.9		41.6	
Histological Grade				
High	31.7	**0.045**	23.94	0.095
Low	68.6		65.28	
Necrosis				
Yes	19.1	**0.003**	23.95	0.138
No	62.4		50.23	
Epithelioid Variant				
Yes	*	*	6.21	**0.036**
No	44.49		43.98	
Bcl-2 protein expression				
Positive	43.2	0.922	26.1	0.679
Negative	46.1		47.4	
Low PI	50.0	0.811	42.5	0.867
High PI	34.3		36.0	
Bcl-x protein expression				
Positive	46.7	0.916	29.5	0.579
Negative	47.2		49.2	
Low PI	46.9	0.803	54.7	0.312
High PI	41.0		27.3	
CC3 protein expression				
Nuclear	54.2		42.5	
Nuclear and cytoplasmic	37.6	0.301	36.0	0.867
Low PI	55.3		56.8	
High PI	14.6	**0.024**	6,9	**< 0.00**

SV, survival; PI, positive index; *, the value could not be calculated because all cases were censored.

al. 1994, Ofner et al. 1995, Yoo et al. 2007, Nadler et al. 2008, Yang et al. 2000). Conversely, in other tumors, such as non-small-cell lung carcinomas, the expression of this protein has been correlated with poor survival (Groeger et al. 2004). Our results showed no correlation between the expression of Bcl-2 and histological grade, as well as no correlation between the expression of Bcl-2 and survival of patients with MPNSTs, similarly to other studies with different tumors. (Hirakawa et al. 1996, Paik et al. 2006, Tsutsui et al. 2006).

Bcl-x is another member of the Bcl-2 proteins family that has been extensively studied in many tumors. Two isoforms, Bcl-x_S and Bcl-x_L, can be generated by alternative splicing of Bcl-x transcripts, having an inductor and inhibition function on apoptosis, respectively (Schoelch et al. 1999). In some malignant neoplasms, higher expression of

TABLE V
Results of the Cox regression model for significant variables in univariate analysis for overall and disease free survival.

Co-variables		p-value	Exp(B)	95,0% CI for Exp(B)	
				Lower	Upper
Overall Survival					
Step 1	Neurofibromatosis type 1	0.448	0.591	0.152	2.301
	Histological Grade	0.831	1.248	0.164	9.529
	Necrosis	0.343	0.468	0.097	2.250
	High PI of CC3	0.469	1.657	0.423	6.497
	Mitotic Index	0.525	1.054	0.895	1.242
Step 2	Presence of NF1	0.417	0.573	0.149	2.196
	Necrosis	0.318	0.451	0.095	2.152
	High PI of CC3	0.472	1.655	0.419	6.543
	Mitotic Index	0.438	1.062	0.913	1.235
Step 3	Presence of NF1	0.465	0.609	0.161	2.301
	Necrosis	0.255	0.413	0.090	1.897
	Mitotic Index	0.289	1.083	0.935	1.254
Step 4	Necrosis	0.143	0.343	0.082	1.434
		0.226	1.093	0.947	1.261
Step 5	Necrosis	0.007	0.199	0.062	0.639
Disease Free Survival					
Step 1	Epithelioid variant	0.705	0.801	0.254	2.528
	High PI of CC3	0.001	7.727	2.194	27.208
Step 2	High PI of CC3	0.002	7.707	2.183	27.210

CI = confidence interval.

anti-apoptotic Bcl-x_L protein was observed, when compared to corresponding normal tissues (Leiter et al. 2000, Guo et al. 2002). In a study with an established NF1-associated MPNST cell line and primary tissue cultured MPNST cells derived from a NF1 patient, high expression of Bcl-x_L was observed (Lee et al. 2012). In another recent study with cell lines, it was found that Bcl-x_L is upregulated in Schwann cells derived from NF1-associated MPNST compared to neurofibromas from NF1 patients and was suggested that this upregulation may be caused by *NF1* deficiency-mediated elevation in Ras signaling. (Park et al. 2013)

Our results showed that there was a higher number of positive MPNSTs for Bcl-x than neurofibromas ($p < 0.001$). Moreover, positive MPNSTs had higher PIs than neurofibromas ($p=0.002$). Interestingly, in a study with many normal tissues, the expression of Bcl-x_L and Bcl-x_S was not observed in Schwann cells, neither in other cells from peripheral nerve sheath (Krajewski et al. 1994). However, in the already mentioned study of Park et al. (2013) with cell lines, the expression of Bcl-x_L in normal Schwann cells could be detected by Western blot, but they presented lower expression when compared to Schwann cells derived from NF1-associated MPSNTs. Because the antibody we used does not distinguish the two isoforms of Bcl-x protein, we cannot know if the expression of Bcl-x observed in the tumors of our study is referred to the anti or pro-apoptotic isoform. Nevertheless, it probably corresponds to Bcl-x_L isoform.

Investigations with other malignant neoplasms demonstrated a correlation between high expression of Bcl-x_L protein with poor prognosis (Friess et al. 1998, Thamboo et al. 2006). Köhler et al. (2002) evaluated the expression of messenger RNA (mRNA) of Bcl-x_L, Bcl-2 and other genes involved in apoptosis in 82 soft tissue sarcomas, including 15 MPNSTs. They concluded that high levels of mRNA of Bcl-x_L were related to a poor prognosis. Unfortunately, this study, as many others, investigated a variety of histological types of soft tissue sarcomas together, and there is no report about the expression of these genes in each type of sarcoma separately. Our research did not show correlation of Bcl-x expression to the prognosis.

In our study, we performed the identification of apoptotic cells through the investigation of the presence of CC3 using IHC. The activation of effectors caspases, like caspase-3, is an event also known as "point of no return" of apoptosis, beyond which the cells certainly will entry to apoptosis (Asselin et al. 2001). The presence of CC3 has been demonstrated in many neoplasms, such as breast cancer, gliomas, neuroblastomas and sarcomas (Konstantinidou et al. 2007, Kobayashi et al. 2007, Matsubara et al. 2008, Nassar et al. 2008).

Double inactivation of *NF1* gene in Schwann cells seems to be the common event in neurofibromas and MPNSTs. (Laycock-van Spyk et al. 2011) This leads to high levels of Ras-GTP, since neurofibromin, which is an important negative regulator of Ras pathway (similar to GTPase-activating protein - GAP) is deficient as a consequence of the *NF1* gene mutation. It is known that Ras activation elicits antiapoptotic effects in many cells and that there is a positive correlation between neurofibromin deficiency and resistance to apoptosis (Dasgupta and Gutmann 2005, Guerrero et al. 2000, Shapira et al. 2007). Therefore, the observation that all neurofibromas and MPNSTs were immunopositive for CC3 in our study was unexpected. Interestingly, a study with cultured Schwann cells from a high-grade MPNST showed high levels of expression of caspase-3 gene (Lee et al. 2004).

The observation of the presence of CC3 in the nucleus and cytoplasm of MPNSTs cells in our study is in accordance with the results of a previous study (Kamada et al. 2005), which demonstrated that this protein is actively translocated, by one of more unknown proteins, from the cytoplasm to the nucleus. The reason why nuclear location of CC3 was only observed in MPNST and not in neurofibromas needs to be further investigated.

In the study with pancreatic neoplasms, using IHC technique and mRNA analysis, the pattern of cellular location of caspase-3 reflected the biological behavior of benign and malignant neoplasms (Satoh et al. 2000). Nuclear location of caspase-3 had statistically significance with the benign characteristic of pancreatic neoplasms, whereas the cytoplasmic location of this protein was correlated to the invasivity of malignant tumors. In some tumors, i.e. meningiomas (Konstantinidou et al. 2007), and Burkitt's and Burkitt-like lymphomas (Nomura et al. 2008), the location of caspase-3 protein has been shown to be a prognostic marker. In a study with 41 sarcomas, but none MPNSTs, the overall and disease free survival were lower in cases of patients with higher levels of CC3 (Matsubara et al. 2008).

Our results showed that high level of CC3 was associated to lower disease free survival. We found that the presence of cytoplasmic staining and high level of CC3 were more frequently associated to high-grade tumors and high mitotic index. Moreover, cytoplasmic location of CC3 was more common in MPNSTs from NF1 patients and high CC3 PI was associated to necrosis. These data show that higher quantities of CC3 are more common in tumors with more aggressive histological features and it is associated with a poor prognosis.

Because CC3 is associated to induction of apoptosis, it would be expected that tumors with high quantity of this protein would have better

prognosis or more indolent features. Nevertheless, we should keep in mind that tumor growth results from lack of balance between proliferation and cell death by apoptosis and our results show that, in more aggressive tumors, although there was high number of apoptotic cells, there was also high number of proliferating cells (high mitotic index).

Finally, our study shows that apoptosis is altered in neurofibromas and mainly in MPNSTs and that the accumulation of Bcl-2, Bcl-x and CC3 may be correlated with the biology of these tumors. Further studies, including other members of Bcl-2 proteins family, such as Bax, Bak, Bad, are required for a better understanding of the alterations in apoptotic mechanisms involved in the biology of neurofibromas and MPNSTs.

RESUMO

OBJETIVOS: Estudar a expressão das proteínas Bcl-2, Bcl-x, assim como a presença da caspase-3 clivada em neurofibromas e tumores malignos da bainha do nervo periférico. A expressão de Bcl-2 e Bcl-x e a presença da caspases-3 clivada foram comparadas com as características clinicopatológicas dos tumores malignos da bainha do nervo periférico e seu impacto nas curvas de sobrevida também foi investigado. MATERIAIS E MÉTODOS: A avaliação das proteínas Bcl-2, Bcl-x e caspases-3 clivada foi realizada em 28 tumores malignos da bainha do nervo periférico e 38 neurofibromas, por imuno-histoquímica, utilizando microarrays de tecidos. A imunoquantificação foi realizada por análise computadorizada de imagem digital. CONCLUSÕES: A apoptose está alterada nos neurofibromas e principalmente em tumores malignos da bainha do nervo periférico. Altos níveis de caspase-3 clivada é mais comum em tumores com características histopatológicas mais agressivas e estão associados com baixa sobrevida livre de doença em indivíduos com tumor maligno da bainha do nervo periférico.

Palavras-chave: proteínas reguladoras da apoptose, tumor maligno da bainha do nervo periférico, neurofibroma, Neurofibromatose 1.

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