Immunoexpression of cell cycle biomarkers in neuroblastoma samples and its correlation with prognostic factors

Imunoexpressão de biomarcadores do ciclo celular em amostras de neuroblastomas e sua relação com fatores prognósticos

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ABSTRACT

Introduction: It is widely known that the expression levels of molecules involved in apoptosis regulation and cell proliferation have prognostic value in patients with neuroblastomas. **Objective:** To determine the expression of Ki67, B-cell lymphoma 2 (BCL-2), phosphatase and tensin homolog (PTEN), BCL-2 associated protein X (BAX) and caspase-8 proteins in neuroblastomas and to propose new prognostic biomarkers that could enable a better classification of risk groups. **Material and methods:** Formalin fixed paraffin embedded neuroblastoma samples ($n = 23$) were arranged into tissue microarray blocks and analyzed by immunohistochemistry. The patients were classified according to clinical and pathological prognostic factors (age, site, presence or absence of bone-marrow infiltration, poorly or well differentiated ganglioneuroblastoma, Schwannian stroma rich or poor, favorable or unfavorable Shimada histology, and presence or absence of MYCN oncogene amplification) and clinical course (with or without fatal outcome, with or without relapses/residual lesion). **Results:** Twelve patients were female; nine children were over 18 months old; nine had extra-abdominal tumors; nine had tumors with unfavorable histology. Fifteen patients underwent bone-marrow biopsy and four were positive for metastasis. Nine patients progressed to fatal outcome. **Conclusion:** Ki67 immunoexpression was lower in cases of Schwannian-stroma rich neuroblastomas ($p = 0.018$) and higher in poorly differentiated cases ($p = 0.013$). PTEN was less positive in stroma rich neuroblastomas ($p = 0.024$). Caspase-8 was more immunopositive in cases of negative bone marrow infiltration ($p = 0.035$). Therefore, these biomarkers could be applied to discriminate groups with poor prognosis.

Key words: neuroblastoma; cell proliferation; apoptosis; prognostic factors.

INTRODUCTION

Neuroblastomas are embryonic neoplasias of the sympathetic nervous system. Besides, it is the most frequent extracranial solid tumor in infancy, commonly diagnosed among children younger than 12 months[14, 18].

Its behavioral diversity, which is sometimes characterized by aggressiveness, suggests that the malignant cell transformation is mostly due to a failure in the signaling pathways that regulate differentiation and cell proliferation[15]. In other occasions, neuroblastomas may present a relatively high benign differentiation rate with spontaneous regression, which may reflect an overactivity of cell differentiation programs and apoptotic cascades. It is particularly worth mentioning those cases with aggressive progression, with early relapses and unfavorable clinical course in spite of a good initial prognosis based on clinical pathological profile. The identification of these patients at diagnosis may allow the implementation of more aggressive therapeutic approaches, even though they are not justified by the initial favorable prognosis, which ultimately would optimize survival rates[18, 27, 30].

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The presence of high levels of proliferative proteins (Ki67) or apoptosis resistance proteins (B-cell lymphoma 2 [BCL-2]) and inhibition of pro-apoptotic proteins (phosphatase and tensin homolog [PTEN], BCL-2 associated protein X [BAX] and caspases) may explain the aggressive behavior of these tumors[14, 40] as well as their chemotherapy resistance[5]. It is acknowledged that the expression levels of molecules involved in apoptosis regulation and cell proliferation such as Ki67, PTEN, caspases, BCL-2 and BAX present prognostic value in patients with neuroblastomas[5, 15]. By analyzing the tumorigenesis process of neuroblastoma and its mechanisms of cell proliferation and apoptosis resistance, we aim to evaluate proliferative and apoptotic biomarkers already reported by the literature (PTEN, Ki67, BAX, BCL-2 and caspase-8), whose immunohistochemical expression at diagnosis may be applied as a tool for the assessment of tumor aggressiveness. Accordingly, this may guide the therapeutic approach and improve its prognosis, which is quite particular[29].

The objective of this study was to evaluate the immunohistochemical expression of proliferative and apoptotic biomarkers already reported by the literature (PTEN, Ki67, BAX, BCL-2 and caspase-8) at the moment of diagnosis as a predictive tool for the assessment of tumor aggressiveness.

MATERIAL AND METHODS

The cases selected for this study comprised paraffin embedded samples of neuroblastomas from pediatric patients of both genders and without neoadjuvant chemotherapy ($n = 23$).

The cases were classified as follows: (a) child's gender; (b) age range at diagnosis (<18 months or ≥ 18 months); (c) lesion site at diagnosis (extra-abdominal or abdominal); (d) infiltrated or non-infiltrated bone marrow; (e) histological differentiation rate (poorly differentiated or in differentiation/ganglioneuroblastomas); (f) clinical course (fatal outcome or relapse/residual disease); (g) type of extracellular matrix (schwannian stroma rich or poor); (h) Shimada classification (favorable or unfavorable histology); (i) presence or absence of MYCN amplification in all cases.

Two tumor areas were prepared for tissue microarray blocks (TMA)[1]. Six sections were performed on the paraffin embedded TMA blocks (4 μm thick) with primary antibodies and incubated for 12 hours in humid chamber at 2 to 8ºC. The following antibodies were applied: anti-PTEN Data Sheet (Novocastra® mouse monoclonal), antiKi67 (Dako® mouse monoclonal), anticaspase-8 (Dako® mouse monoclonal), antiBCL-2 (Dako® mouse monoclonal) and antiBAX (Dako® mouse monoclonal). Advance Dako® was used as a secondary antibody, which was incubated for 30 minutes at 2 to 8ºC. 3-3'-diaminobenzidine complex (DAB) + Dako® substrate was added on electrically charged Star Frost® slides and counterstaining was carried out with Harris hematoxylin.

The slides immunostained with Ki67 and PTEN were analyzed through counting of positive nuclei per high power field (HPF) at optical microscopy (BX40 Olympus® microscope) with 40× objective. There were six HPF per case.

The slides immunostained with BCL-2, caspase-8 and BAX were assessed through BX40 Olympus® microscope and Image Pro Plus® program with DELL® computer and Dino-eye® camera. The areas of immunopositivity for caspase-8, BCL-2 and BAX were identified with amplification of 400× HPF and image analysis software. The program determined the total immunopositive area in square micrometer (um^2) per HPF. Eight HPF were analyzed per case in order to establish the mean immunopositivity area per antibody (caspase-8, BCL-2 and BAX).

The assessment of MYCN amplification was undertaken through fluorescence in situ hybridization (FISH) at Hospital A. C. Camargo in São Paulo. We applied commercial probe (LSI SYT Dual Colour Break Apart Rearrangement Probe Vysis Abbott Laboratories UK) on electrically charged slides containing two micrometer TMA sections with neuroblastoma samples after deparaffinization, pre-treatment, heating and digestion with proteinase according to Vysis kit[17].

The statistical analysis considered data descriptive measures, including frequencies and percentages for categorical variables, mean, median, minimum values, maximum values and standard deviation for quantitative measures. Student's t test and non parametric Mann-Whitney test were used for comparison of groups defined by clinical pathological variables and clinical course in relation to quantitative variables. As to dichotomous nominal variables, the groups were compared through Fisher's exact test. The association level between two markers was calculated by Spearman's rank correlation coefficient and by Kappa coefficient. Kaplan-Meier curves were build to assess survival rates and comparison between curves was conducted by log-rank test. p values < 0.05 showed statistical significance. We employed SPSSV8® statistical analysis program.

Receiver operating characteristic (ROC) curves were adjusted to determine the cut-off points that could best discriminate the relation between two histological groups (poorly differentiated and in differentiation/ganglioneuroblastoma). This determination enabled the separation of samples into two groups: (a) high immunopositivity; (b) low immunopositivity. Immunopositivity for Ki67 was considered high when there were more than 159 positive nuclei per HPF and low when there were less than 159 positive nuclei per HPF. Immunopositivity for PTEN was considered high when there were more than 637 positive nuclei per HPF and low for less than 637 positive nuclei per HPF.

RESULTS

There was no significant prevalence between male and female genders. Most patients were diagnosed with neuroblastoma with less than 18 months of age (60.8%) and most lesions were abdominal (60.8%) with well differentiated histological rate (60.8%). Only 65% had undergone bone marrow biopsy and 26.6 of which had infiltrated bone marrow. At follow-up, three years after diagnosis 60% of patients were alive, though 57% still had the disease (**Table 1**).

Ki67 and PTEN immunoexpression was related to the type of tumoral stroma, noting that both biomarkers presented higher immunopositivity in stroma poor lesions ($p = 0.018$ and $p = 0.024$, respectively in the Table 1).

TABLE 1 – Correlation between mean biomarker values per HPF and the studied clinical pathological variables

Variable		n	Ki67	PTEN	Caspase-8	BCL-2	BAX
	Female	12	188.78	475.49	41 668 619.83	4 271.71	32 939 678.76
	Male	11	183.76	649.58	37 843 054.17	5 843.30	25 279 502.44
Diagnosis	≤ 18 months	14	153.81	526.37	38 878 699.41	5 005.19	30 434 898.82
	> 18 months	9	237.04	609.11	41 332 804.68	5 133.07	27 473 565.38
Site	Extra-abdominal	9	197.63	649.93	38 850 179.00	4 996.59	24 460 117.90
	Abdominal	14	179.14	500.13	40 474 673.06	5 099.68	32 372 115.06
Bone Marrow	Negative	11	189.15	524.29	**41 411 751.7***	4 882.30	32 089 747.91
	Positive	4	205.17	563.83	**41 338 283.5***	3 921.56	36 510 520.00
Differentiation	Low	9	276.00	724.74	39 761 505.47	5 725.10	37 807 537.58
	High	14	128.76	452.04	39 888 820.33	4 595.33	23 791 630.98
Shimada	Favorable	18	146.56	522.81	39 096 447.3	4 882.79	26 532 881.30
	Unfavorable	5	329.73	688.13	42 512 196.63	5 651.56	39 151 761.70
Stroma	Poor	9	**276.00***	**724.74****	38 518 567.52	5139.15	42 853 898.15
	Rich	14	**128.76***	**452.04****	39 888 820.33	4 595.33	23 791 630.98
Deaths	Survival	5.45 years	192.96	685.11	43 099 546.44	4 947.69	24 848 126.88
Alive	Follow-up 1.3 years	14	182.14	477.51	37 742 936.85	5 133.53	32 122 680.72
With relapse/ residual	Follow-up 3 years	8	155	350.89	37 161 213.84	5 128.72	24 074 267.64
Without relapse/ residual	Follow-up 3 years	6	218.33	646.32	38 518 567.52	5 139.15	42 853 898.15

Ki67 and PTEN immunopositivity results are expressed in number of positive nuclei per HPF (mean value per 6 HPF). BCL2, caspase-8 and BAX immunopositivity results are expressed in positivity area per HPG in square micrometer (mean value per 8 HPF). The statically significant differences in these comparisons are in bold types (* p = 0.035; ** p = 0.018; *** p = 0.024).

HPF: high power field; PTEN: phosphatase and tensin homolog; BCL-2: B-cell lymphoma 2; BAX: BCL-2 associated protein X.

As to BCL-2 and BAX, there were no statically significant differences between mean immunopositivity of these antibodies in comparison with groups defined by clinical pathological variables and clinical course. Caspase-8 showed higher immunopositivity in cases with negative bone marrow in comparison with positive bone marrow ($p = 0.035$) (Table 1).

The cutoff points allowed new comparisons with groups defined by clinical pathological variables and clinical course (**Tables 2**, **3** and **4**).

High immunopositivity for Ki67 protein ($p = 0.013$) demonstrated statically significant correlations with poorly differentiated neuroblastoma. The correlation between stroma pattern and ROC curves of Ki67 and PTEN was also evaluated. The high immunohistochemical expression of Ki67 (>159 positive nuclei per HPF) and PTEN (> 637 positive nuclei per HPF) was associated with Schwannian stroma poor cases ($p = 0.012$ and $p = 0,042$, respectively).

The correlation between BCL-2, BAX and caspase-8 cutoff points and groups defined by clinical pathological variables and clinical course did not reveal statically significant results.

Following the clinical course analysis, there was no statistically significant correlation between fatal outcome/relapse rates and the surveyed biomarkers. There was a tendency to statistical significance in the correlation within low PTEN rates (< 637 positive nuclei per HPF) and high rates of post-chemotherapy residual disease ($p = 0.070$).

MYCN proto-oncogene amplification was conducted by FISH method and it was not observed in any of the cases from this study (**Figure**).

DISCUSSION

Most patients were diagnosed at less than 18 months old (60.86%), which is commonly described in the literature. Although some authors claim there is a slight prevalence of the male gender (1.2 boys: 1 girl), there was no significant prevalence of gender in this study. It was observed a considerable percentage of cases with infiltrate bone marrow and extra-abdominal disease, that is to say, advanced clinical stages that aggravate the prognosis, as it is described in the literature[5]. Epidemiological data demonstrate that neuroblastomas present clinical pathological aspects that have a negative influence on the answer to the treatment, which increases the probability of chemoresistance and low survival rates.

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TABLE 2 – Correlation between Ki67 and the studied clinical pathological variables

Variable	Ki67 immunopositivity		Total	p value*
	> 159 (high)	≤ 159 (low)		
Age at diagnosis				
≤ 18 months	5 (35.71%)	9 (64.29%)	14	
> 18 months	5 (55.56%)	4 (44.44%)	9	0.417
Lesion site				
Extra-abdominal	4 (44.44%)	5 (55.56%)	9	
Abdominal	6 (42.86%)	8 (57.14%)	14	1.000
Bone marrow Status				
Negative	5 (45.45%)	6 (54.55%)	11	
Positive	3 (75.00%)	1 (25.00%)	4	0.569
Shimada classification				
Unfavorable	4 (80.00%)	1 (20.00%)	5	
Favorable	6 (33.33%)	12 (66.67%)	18	0.0886
Lesion differentiation				
Poorly differentiated	7 (77.78%)	2 (22.22%)	9	
Highly differentiated	3 (21.43%)	11 (78.57%)	14	0.013

Ki67 mean immunopositivity was assessed by the number of positive nuclei per HPF for each case.
*: Fisher's exact test, p < 0.05; HPF: high power field.

TABLE 3 – Correlation between PTEN biomarker and the studied clinical pathological variables

| Variable	PTEN immunopositivity		Total	p value*
	> 637 (high)	≤ 637 (low)		
Age at diagnosis				
≤ 18 months	5 (35.71%)	9 (64.29%)	14	
> 18 months	4 (44.44%)	5 (55.56%)	9	1.000
Lesion site				
Extra-abdominal	6 (66.67%)	3(33.33%)	9	
Abdominal	3 (21.43%)	11(78.57%)	14	0.077
Bone marrow status				
Negative	4 (36.36%)	7 (63.64%)	11	
Positive	1 (25.00%)	3 (75.00%)	4	1.000
Shimada Classification				
Unfavorable	3 (60.00%)	2 (40.00%)	5	
Favorable	6 (33.33%)	12 (66.67%)	18	0.283
Lesion differentiation				
Poorly differentiated	6 (66.67%)	3 (33.33%)	9	
Highly differentiated	03 (21.43%)	11 (78.57%)	14	0.077

PTEN mean immunopositivity was assessed by the number of positive nuclei per HPF for each case.
*: Fisher's exact test, p < 0.05; PTEN: phosphatase and tensin homolog; HPF: high power field.

TABLE 4 – Correlation between the neoplasia stroma pattern (Schwannian stroma rich or poor) and Ki67
and PTEN ROC curves calculated by exact Fisher test

	p value	Hypothesis
Ki67 Fisher's exact test	0.012	Stroma rich cases may have low Ki67 values and stroma poor cases may have high Ki67 values
PTEN Fisher's exact test	0.042	Stroma rich cases may have low PTEN values and stroma poor cases may have high PTEN values

ROC: receiver operating characteristic; PTEN: phosphatase and tensin homolog.
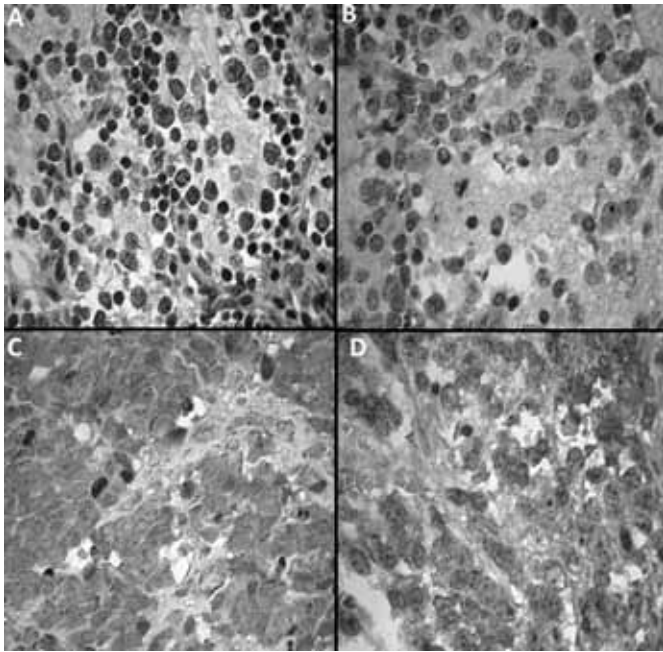
FIGURE – *Immunopositivity of neuroblastoma biomarkers. A) PTEN: nuclear positivity; B) BCL-2: perinuclear positivity; C) caspase-8: cytoplasmic positivity; D) BAX: cytoplasmic positivity. 400×*
PTEN: phosphatase and tensin homolog; BCL-2: B-cell lymphoma 2; BAX: BCL-2 associated protein X.

Ki67 is regarded as one of the most efficient proliferation markers currently surveyed. There is a strong correlation between its high positivity and a more particular prognosis in several tumors such as melanomas, astrocytomas, breast cancer and squamous cell carcinomas[8, 16, 35-37, 41]. Researches have demonstrated that the higher Ki67 expression, the worse the prognosis is. Therefore, high Ki67 expression would reveal those cells with higher proliferation potential and highly aggressive behavior. When Ki67 expression was analyzed in neuroblastoma samples and each clinical pathological variable was compared, it was noted that there was an inverse correlation (statiscally significant) between this proliferative marker (Ki67) and tumoral differentiation, inasmuch as well differentiated lesions had lower Ki67 rates. Additionally, there was a statiscally significant correlation between Ki67 and stroma pattern of the lesions, insofar as the expression of this proliferative marker (Ki67) was higher in stroma-poor cases. However, it was not possible to establish a direct relation with other poor prognostic variables (age, bone marrow infiltration, extra-abdominal disease, Shimada classification and MYNC amplification). Some authors have unsuccessfully attempted to establish a correlation of Ki67 with malignity level of astrocytic neoplasias and clinical factors such as gender and age. On the other hand, they have demonstrated the correlation between proliferation rate caused by Ki67 and malignity level of the lesions[6]. Analyzing Ki67 expression in neuroblastomas, Korja and Graham observed an association between high rates of the marker and tumors classified as undifferentiated, which evinces an increase in the proliferative index in cases with poor prognosis[11, 17]. Several authors claim that this marker is a relevant proliferative index and it can be incorporated into the diagnostic routine of several tumors to evince tumoral aggressiveness despite the fact it cannot be considered a prognostic predictor solely[8, 16, 17, 36, 41]. As it is corroborated by other researchers, in our study the high expression rates of this protein were statiscally related to tumors with unfavorable histology. Ki67 may be a marker of low differentiation and poor clinical evolution in neuroblastomas, which aids the stratification of more aggressive tumors.

PTEN was another surveyed marker. Its high rate was observed in patients with extra-abdominal lesions and with low differentiated tumors, demonstrating a statistical tendency in these cases. Furthermore, PTEN immunohistochemical expression rates were higher in cases with stroma- poor, which would have poor prognosis. These findings seem to be contrary to those observed by other studies, in which high PTEN expression rates were generally linked with good prognostic factors[3, 16], inasmuch as PTEN is a tumor suppressor gene. Nevertheless, the changes in its signaling pathways (mainly phosphatidylinositide 3-kinases [PI3-K]) would cause a modification in the normal cell apoptosis pathway.

PTEN also seems to operate as cell cycle controller by avoiding excessive proliferation[30, 33, 39, 42]. Low PTEN expression commonly leads to an unbalance in the cell regulation function and, therefore, a worse progression, which is evinced in tumors such as glioblastomas and pancreas carcinomas, apart from neuroblastomas[10, 13, 22, 30, 38, 39]. The fact that we had higher PTEN immunohistochemical expression in stroma-poor cases (poor prognosis according to Shimada) could represent solely an epiphenomenon, that is to say, PTEN protein expression would be high since the proliferation rates of these tumors would be much higher and PTEN would operate as cell cycle regulation and apoptosis inductor, inasmuch as undifferentiation promotes proliferation and apoptosis. As neuroblastomas differentiate, both proliferation and apoptosis rates tend to reduce as well as PTEN expression. It is widely acknowledged in the literature that poorly differentiated neuroblastomas have high apoptosis rates (represented by high mitosis/karyorrhexis indexes). The latter would be a criterion proposed by Shimada for the classification of these tumors into favorable and unfavorable histology. Moreover, PTEN marker exhibited a tendency to correlation with residual disease, considering that the cases with low PTEN immunopositivity presented high rates of post-chemotherapy residual disease ($p = 0.070$), which corroborates the hypothesis that low PTEN immunoexpression may represent a poor response to the treatment.

BCL-2 gene family, namely pro-apoptotic (BAX) and anti-apoptotic (BCL-2) molecules, play a crucial role in apoptosis regulation, insofar as they directly activate caspase pathways[4, 6, 9, 12, 23, 24, 26, 28]. The analysis of these markers in the selected neuroblastoma samples revealed no statistically significant correlation neither among them, nor among the studied clinical and pathological variables. Subramaian *et al.*[34] noted that drugs that activate BAX expression provided a better cytotoxic response in neuroblastomas and ganglioneuromas. Chesler *et al.* investigated the relation between chemoresistance and BCL-2 and BAX

expression in neuroblastomas and high BCL-2 rates were linked with high chemotherapy resistance rates[7, 19].

As to caspases, in our study there was no statiscally significant correlation when we compared immunohistochemical caspase-8 results with clinical pathological variables, except when we analyzed caspase-8 immunopositivity with cases in which there was no bone marrow infiltration. The expression of this marker was higher in these samples. Caspases are widely acknowledged by its importance in programmed cell death and several studies correlate caspase inhibition, mainly caspase-8, with neoplasias such as adenocarcinomas, pulmonary carcinomas and neuroblastomas[21, 25, 31, 35]. Caspase-8 is a pro-apoptotic protease and in neuroblastomas it would induce programmed cell death, hence its relation with better prognosis, as it was substantiated in our study. Nevertheless, this protein also promotes the migration of non-apoptotic cells and may contribute to metastases when apoptosis is impaired. Caspase-8 interacts with multi-protein complexes that include focal adhesion kinase (FAK) and calpain 2 (CPN2), it increases cleavage of FAK substrates and it also promotes cell migration. The absence of caspase-8 expression may be linked with chemotherapy resistance[2, 20, 31, 32]. Thus, in our study this protein was associated with patients with better prognosis, which is also corroborated by the literature.

CONCLUSION

Ki67 immunopositivity revealed statiscally significant correlation with stroma- rich neuroblastomas, considering that it was less positive in these cases ($p = 0.018$). Furthermore, it also showed high immunopositivity in cases with poorly differentiated and stroma- poor neuroblastomas ($p = 0.013$ e 0.012, respectively).

PTEN positivity exhibited statiscally significant correlation with stroma- rich neuroblastomas and it was less positive in these cases ($p = 0.024$). Its high immunopositivity was more frequent in stroma- poor cases ($p = 0.042$).

Caspase-8 was more positive in patients with negative bone marrow.

The authors believe that these biomarkers could be employed to aid the identification of those patients that, in spite of belonging to low risk groups, may progress to a worse prognosis.

RESUMO

Introdução: Sabe-se que os níveis de expressão de moléculas envolvidas na regulação da apoptose e da proliferação celular apresentam valor prognóstico em pacientes com neuroblastomas. Objetivo: Avaliar a imunoexpressão das proteínas Ki67, B-cell lymphoma 2 (BCL-2), phosphatase and tensin homolog *(PTEN), caspase-8 e BCL-2* associated protein *X (BAX) em neuroblastomas na tentativa de propor novos biomarcadores prognósticos que poderiam auxiliar na melhor discriminação dos grupos de risco. Material e métodos: Amostras de neuroblastoma (*n = 23*) foram submetidas à técnica* tissue microarray *e analisadas com imuno-histoquímica. Os pacientes foram classificados de acordo com os fatores prognósticos clínico-patológicos (idade, localização, medula óssea infiltrada ou não, pouco diferenciado ou em diferenciação/ganglioneuroblastoma, rico ou pobre em estroma com células de Schwann, histologia favorável ou desfavorável segundo Shimada, presença ou não da amplificação do MYCN) e com o curso clínico (se em óbito ou vivo, com ou sem lesão residual/recidiva). Resultados: Doze casos eram do sexo feminino; nove tinham idade acima de 18 meses; nove apresentavam tumores extra-abdominais; e nove cursavam com histologia desfavorável. Quinze pacientes foram submetidos à biópsia de medula óssea, sendo quatro com apresentação de metástase. Nove pacientes evoluíram ao óbito. Conclusão: A imunoexpressão do Ki67 foi mais baixa nos casos ricos em estroma (*p = 0,018*) e elevada nos casos pouco diferenciados (*p = 0,013*). O PTEN apresentou-se menos positivo em neuroblastomas ricos em estroma (*p = 0,024*). A caspase-8 foi mais imunopositiva em casos com medula óssea negativa (*p = 0,035*). Esses biomarcadores poderiam ser utilizados para auxiliar a discriminar grupos de pacientes de pior prognóstico.*

Unitermos: neuroblastoma; proliferação celular; apoptose; fatores prognósticos.

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