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Original article

Immunohistochemistry of apoptosis-related proteins in retinoblastoma

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ABSTRACT

Retinoblastoma is the most common intraocular malignant neoplasia during childhood and results from the partial or total inactivity of the retinoblastoma protein (pRb). In the absence of pRb, the E2F transcription factors increase the levels of cell cycle proteins as well as some pro-apoptotic proteins. We intended to study the immunohistochemistry profile of apoptotic-related proteins in retinoblastoma. We also evaluated the association between the expression of apoptotic protein and stage of tumor or survivor after a 5 year follow up. Apoptosis-related proteins (Apaf-1, Bak, Bax, Bcl-2, Bcl-xL, Bim-long, MDM2, p53, pro-caspase-3, PUMA, Smac/DIABLO and cleaved caspase-3) were evaluated using immunohistochemistry on tissue microarrays which contained samples of retinoblastoma tumors taken from ninety-three patients without any treatment previous to surgery. The immunohistochemistry reactions were evaluated using an optical microscope as well as the ACIS III® platform. The pro-apoptotic proteins (APAF-1, Bax, p53, PUMA, Smac/DIABLO) were more frequently expressed than the anti-apoptotic proteins (Bcl-2, Bcl-xL and MDM2). The protein Bcl-xL had a negative correlation with cleaved caspase-3, a marker of cell apoptosis. Bcl-xL may be implicated in an apoptosis block.

1. Introduction

The Retinoblastoma is the most common intraocular malignancy in childhood and usually originates from the complete or partial loss of function of pRb protein. This protein is encoded by the gene RB1, and performs numerous important functions in the cell. The main function is the control of the cell cycle at the transition G1/S and the loss of this protein results in unregulated cell proliferation [1].

The pRb has also an important role in apoptosis. The transgenic mice embryos lacking two copies of the RB1 gene have serious abnormalities in central nervous system and hematopoiesis due to the intense and aberrant apoptosis in these tissues causing death of the embryo before birth [2]. However, it was observed that placentas of these mice showed alterations in their architecture and function. Moreover, embryos RB1-/- with normal placenta (RB1+/+) showed less aberrant apoptosis. It seems that in the absence of pRb, cells would become more sensitized to apoptosis by extracelular stimulus, like ischemia, caused by those alterations in placenta [3].

In the cell, pRb exerts its function by binding with the transcription factors E2F family. In the absence of pRb, E2F transcription factors are released and activate transcription of pro-apoptotic proteins as apoptotic activation factor-1 (APAF-1) [4] and pro-caspases [5]. Moreover, E2F binds to p53 and increases p53-dependent transcription of Bax, APAF-1, caspases and inhibits transcription of Bcl-2 [6].

In many tissues, loss of pRb induces expression of large amounts of p14ARF that inhibits the function of anti-apoptotic proteins MDM2 and MDM4 and activates p53-dependent apoptosis [7].

Apoptosis is often defined as a form of cell death that involves an activating sequence of proteins called caspases [8]. It is character-

Abbreviations: pRb, retinoblastoma protein; AI, apoptotic index; IAP, inhibitors of apoptosis proteins.

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ized by morphological changes in cells as fragmentation of nucleus, membrane blebbing and formation of apoptotic bodies.

Few studies quantified the apoptosis in retinoblastoma and correlated it with prognostic factors. KERIMOGGLU et al. [9] measured apoptotic index by TUNEL technique. They obtained a significant correlation between apoptotic index (AI) and metastasis, and AI and tumor size. A significant inverse correlation between AI and proliferative index was observed [9]. TATLIPINAR et al. [10] found no association between AI with variables such as prior treatment, degree of tumor differentiation, and unilateral vs. bilateral tumors. Nevertheless, Sitorus at al. [11] demonstrated a positive correlation between AI and mitotic index. Caspase-3 was expressed in most samples. These findings suggest that apoptosis is triggered mainly by the intrinsic pathway in the retinoblastoma.

Describing the immunohistochemical expression of apoptosis-related proteins could improve our understanding about retinoblastoma. Our study intended to demonstrate prognostic factors for retinoblastoma and a block of the apoptotic cascade which makes the tumor more resistant to apoptosis.

2. Material and methods

2.1. Casuistic

We included 93 patients with retinoblastoma who underwent enucleation as a first treatment between 1986 and 2000 at the A. C. Camargo Hospital-São Paulo city, Brazil. In patients with both eyes enucleated, only the first eye was included. Clinical data such as age, sex, time of referral, bilaterality and family history were collected from medical records and we considered as loss of follow-up patients whose last information was prior to two years.

2.2. Tissue microarray

Tissue microarrays built with a tissue microarrayer (Beecher Instruments, Silver Spring, MD) were used for immunohistochemical assays. We selected tumor areas of approximately 1 mm^2 from samples of retinoblastoma fixed in buffered formalin and stored in paraffin blocks. Necrosis, calcifications and areas of invasion of choroid where melanin could interfere with the reading of the immunohistochemistry reaction were avoided.

2.3. Antibodies

We tested 12 different primary antibodies (Appendix). These antibodies are markers of protein expression of intrinsic and common apoptotic pathway. The immunohistochemical reaction was evaluated by two different methods: a qualitative and a quantitative method. In the qualitative method, the pathologist classified samples as positive if the intensity of immunohistochemistry was equal to or greater than moderate, or more than 10% of the cells were stained. A semi-automated method using Dako ACIS III ® Image Analysis System was designed in the quantitative method. A tumoral area (minimum of 0.5 mm^2) without necrosis or calcification was drawn by the pathologist (RJMN). For markers which stained in a cytoplasmic pattern, the software version 3.0.1 ACIS "Cytoplasmic Application" was used and a percentage of stained pixels and the mean of intensity of staining were displayed For nuclear markers, the ACIS Nuclear Application was the choice and in this application, the percentage of stained nuclei and the average of intensity of staining weredisplayed. The product of percentage and intensity of staining were the measure considered for the correlation study.

The apoptotic index was measured by counting stained cells in a thousand tumor cells using Axion Vision 3.1(Carl Zeiss Vision-Germany)

2.4. Pathological characteristics

All slides were reviewed by a pathologist (RJMN). Optic nerve, eye coats and anterior chamber invasion were evaluated as well as the presence of extra ocular disease. The differentiation of retinoblastoma was classified according to the presence of Flexner-Winterstein rosettes. Tumors formed by more than 80% of those rosettes were classified as well differentiated. Tumors that showed no formation of rosettes were classified as poorly differentiated and the others were considered moderately differentiated. Presence of choroid invasion was considered by the presence of tumor beyond the Brunch membrane [12]. Focal invasion was characterized by up to three microscopic foci beyond this membrane, and massive invasion by more than three microscopic foci or macroscopic invasion. Sclera invasion was characterized by tumor dissection of scleral tissue. Optic nerve invasion was assessed in longitudinal cut and was classified as pre-laminar invasion, laminar invasion and post-laminar invasion [13]. Involvement of the resection margin of the optic nerve by tumor was also referred. Impairment of anterior chamber was shown by tumor cells anterior to lens or iris.

2.5. Statistical analyses

The data analysis was performed using the statistical program Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to assess whether a variable had a normal distribution. The Chi-square test or Fisher's exact test (when at least one of the expected frequencies was less than 5) were used for the combination of variables. The association of non-categorical variables was analyzed by the student *t*-test or Mann-Whitney test when distribution was not normal. For the correlation of variables, we applied the Pearson correlation when there was a normal distribution and the Spearman correlation for the others. Survival analysis was performed using the Kaplan-Meier and comparisons between the curves were made using the log-rank test. A correlation study between manual quantification and ACIS III quantification was done. A kappa index was calculated for each immunohistochemistry of apoptosis-related protein. An error $\alpha = 5\%$ was first established but then we corrected using Bonferroni due to multiple comparisons.

3. Results

The observation of immunohistochemistry showed a predominance of expression of pro-apoptotic proteins. APAF-1, Bax, p53, PUMA and Smac/DIABLO were expressed in most of the samples (Table 1). Only one anti-apoptotic protein, the Bcl-xL, was moderately expressed. Bcl-2 and MDM2 were present in a few samples.

The cleaved caspase-3, that marks cells in apoptosis, stained diffusely some samples of retinoblastoma, while in others there were few stained cells (Fig. 1). The cleaved caspase-3 equally stained cells in well and poorly differentiated retinoblastoma. However, there were more stained cells in boundary areas between necrosis and viable tumor (Fig. 1). The average of AI was 0.12; however, more than 30% of the tumors were less apoptotic index than 0.05. (Fig1G)

The p53 protein showed different forms of expression. Overall, p53 was diffusely expressed in retinoblastoma. The p53 was demonstrated in well or poorly differentiated tumors, in areas with or without apoptotic cells and in tumor cells close or far to blood vessels. One interesting pattern was an increase of intensity in cells at the limit between viable tumor and necrosis (Fig. 2). Bax protein showed diffuse cytoplasmatic expression in most cells