Int. J. Morphol.,
30(3):1029-1034, 2012.

Immunohistochemistry Analysis of Proteins Related with Apoptosis as Prognostic Factor in Epidermoid Carcinoma of Penis

Análisis Inmunohistoquímico de Proteínas Relacionadas con la Apoptosis como Factor Pronóstico en el Carcinoma Epidermoide de Pene

[*]Novais, P. C.; [**]de Paula, A. A. P.; [***]Reis, R. B.; [*]Barione, D. F.; [**]Franqueiro, A. G.; [*]Rodrigues Jr., A. A.; [*]Lizarte, F. S. N.; [*]de Carvalho, C. A. M.; [***]Tirapelli, D. P. C.; [*****]Peria, F. M.; [****]Saggioro, F. P.; [******]Tucci Jr., S.; [***]Cologna, A. J.; [*******]Martins, A. C. P. & [***]Tirapelli, L. F.

NOVAIS, P. C.; DE PAULA, A. A. P.; REIS, R. B.; BARIONE, D. F.; FRANQUEIRO, A. G.; RODRIGUES JR., A. A.; LIZARTE, F. S. N.; DE CARVALHO, C. A .M.; TIRAPELLI, D. P. C.; PERIA, F. M.; SAGGIORO, F. P.; TUCCI JR., S.; COLOGNA, A. J.; MARTINS, A. C. P. & TIRAPELLI, L. F. Immunohistochemistry analysis of proteins related with apoptosis as prognostic factor in epidermoid carcinoma of penis. ***Int. J. Morphol., 30(3)***:1029-1034, 2012.

SUMMARY: The aim was to analyze the protein expression of apoptotic genes caspase-3, caspase-8 and bcl-2 with the immunohistochemistry technique, correlating with tumor grade (I, II and III) and with the patient survival in order to understand the basic mechanism of tumoral transformation. The immunohistochemistry reactions on 50 samples of squamous cell carcinoma were carried out with the avidin-biotin immunoperoxidase method and antigen recovery. The analyses were made using the graduation method "in crosses" (0 to 4 crosses - no stain to more than 75% of positives cells) and in categories (low, intermediate, high) of the cytoplasm immunoreactivity of the epidermoid penile carcinoma cells. It was observed a statistically significant difference when the expression of caspase-3 were compared with the grades I and II of the tumor (p=0.0010) and when comparing the patient survival with the grades I and II of the tumor (p=0.0212). The protein bcl-2 was more expressed than caspase-3 and caspase-8 proteins, suggesting that the apoptotic rate in this carcinoma is low. The higher expression of the anti-apoptotic protein bcl-2 suggests a higher preservation of the tumoral cells.

KEY WORDS: bcl-2; Caspase-3; Caspase-8; Penile cancer; Apoptosis.

INTRODUCTION

The penile cancer is considered a rare condition in Europe, where about 4000 cases (0.5% of all cancers) are diagnosed each year. However, in someregions such as Asia, South American, and Africa this disease represents 10 to 20% of all malign tumors in men (Mosconi *et al.,* 2005).

The ideal treatment of this pathology is controversial. Frequently, the primary lesion is treated with radical surgical procedure or curative surgery by partial or total penectomy. Even though the amputation reaches a very high rate of control, it causes loss of urinary control and normal sexual function, which is associated with psychological problems in a large number of treated patients (Harden & Tan, 2001).

Hanahan & Weinberg (2000) suggest that six essential alterations in cell physiology dictate the malignant growth. They are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis.

Recently, evidences have shown that the evasion of apoptosis is an important event in carcinogenesis. The failure in this process occurs due to molecular alteration. Not only can the identification of these changes provide a better understanding about the biological mechanism of

[*] Postgraduate Student-Doctorate, Department of Surgery and Anatomy, Ribeirão Preto Faculty of Medicine, University of São Paulo (USP), SP, Brazil.
[**] Oncourology Division Araújo Jorge Hospital, Association of Cancer Combat of Goiás, Goiânia, Brazil.
[***] MD, PhD, Doctor Professor -Department of Surgery and Anatomy, Ribeirão Preto Faculty of Medicine, University of São Paulo (USP), SP, Brazil.
[****] Postgraduate Student-Doctorate, Department of Pathology, Ribeirão Preto Faculty of Medicine, University of São Paulo (USP), SP, Brazil.
[*****] PhD, Doctor Professor- Department of Clinical Medicine, Ribeirão Preto Faculty of Medicine, University of São Paulo (USP), SP. Brazil.
[******] PhD, Associated Professor, Department of Surgery and Anatomy, Ribeirão Preto Faculty of Medicine, University of São Paulo (USP), SP, Brazil.
[*******]Full Professor and Head, Department of Surgery and Anatomy, Ribeirão Preto Faculty of Medicine, University of São Paulo (USP), SP, Brazil.

apoptosis, but they can also foresee the clinical behavior and induce therapeutic interventions (Wang *et al*., 2004).

Immunohistochemical techniques and other methods have been used in order to analyze the alteration in the products of genes to predict the disease behavior. Unfortunately, as penile cancer is very rare, there are not a large number of studies about the genesis and the progression of this disease (Guimarães *et al.*, 2007). It is important to find a reliable non-invasive method to predict the prognostic of the disease and determine less aggressive procedures.

MATERIAL AND METHOD

The protocol was approved by The Ethic Committee in Research at HCFMRP/SP. During the study, 50 cases of human epidermoid carcinoma of penis were analyzed. The samples were retrospectively collected by resection surgery between 1992 and 2005 and archived at the Laboratory of Pathology, Hospital Araújo Jorge - Goiânia-GO. The pT classifying system was used in order to stage the penile cancer (Table I) and the Broders system for the histological grade (G1- well differentiated, G2- moderately differentiated and G3- undifferentiated).

Paraffin blocks were submitted to 3mm cuts in Reichert Jung 2040 microtome. Later, the immunohistochemical reactions were conducted using the avidin-biotin imunoperoxidase technique and antigen recovery performed under wet heating in a pressure cooker (Optisteam Plus) (Taylor *et al*., 1994). Slides were incubated with the primary antibody, mouse caspase-3 (CPP32), clone JHM62 (1:200 - liofilisate monoclonal, Novocastra®); mouse caspase-8 (CL-CASP-8), clone 11B6 (1:100 - liofilisate monoclonal, Novocastra®); and mouse bcl-2 (NCL-bcl-2-486), clone 3.1 (1:200 - liofilisate monoclonal, Novocastra®). Endogenous peroxidase was blocked with H2O2 at 3%. Reactions were always compared with a positive control, human amygdale for caspase-3 and bcl-2, and human testicle for caspase-8.

The slides were analyzed using Zeiss® microscope, model Axioskop 2 plus, in a high-power field (X400). The images were registered by Axio Cam Hrc® attached in the microscope, and filed by Axio Vision 4.6® software.

To evaluate the protein expression, the areas with the higher concentration of positive cells, or stained cells, in each slide were selected. The method used for counting the positive cells was the "in crosses" quantification of cytoplasm immunoreactivity in tumor cells. It was calculated the percentage of positive neoplasic cells in an average of 1000 cells counted (positives and negatives).

The fields were evaluated according to the percentage of stained cells and graduated as 0 (zero) no stain; 25% + (1 cross) low expression; from 26% to 50% ++ (2 crosses) moderate expression; from 51% to 75% +++ (3 crosses) high expression; and above 75% of stained cells ++++ (4 crosses) high expression.

The immunostained pattern for the target proteins were analyzed alone and compared with the tumor grade and patient survival rate; and showed an average of normal distribution. The patient data were obtained from the medical records and the analyses took into account the period between the surgery and the last consult (months). Data were analyzed with One-Way ANOVA method and Pos-test of Tukey when comparing the protein expression and the tumor grade. Survival curves were built using the Logrank test and compared with non-parametric Qui-square test to compare the survival rate with the tumor grade and expression. Values of $p<0.05$ were considered statistically significant.

RESULTS

After semi-quantitative analysis it was noticed that the intensity of caspase-3 was high (Fig. 1A) in 3 cases (+++ and ++++), in 9 cases it was intermediary (Fig. 1B) (++), and it was low in 38 cases (0 and +). The relationship between the stained cell percentage with caspase-3 antibody and the penile tumor

Table I. pT grading system of penile cancer.

PT Grading	Local Commitment
pTo	No primary tumor
pTis	*in situ*
pTa	Verrucous
pT1	Restrict to epithelium and subepithelial conjunctive tissue
pT2	*Cavernosus corpus* and *spongiosum corpus* invasion
pT3	Urethra or prostate invasion
pT4	Other structures invasion

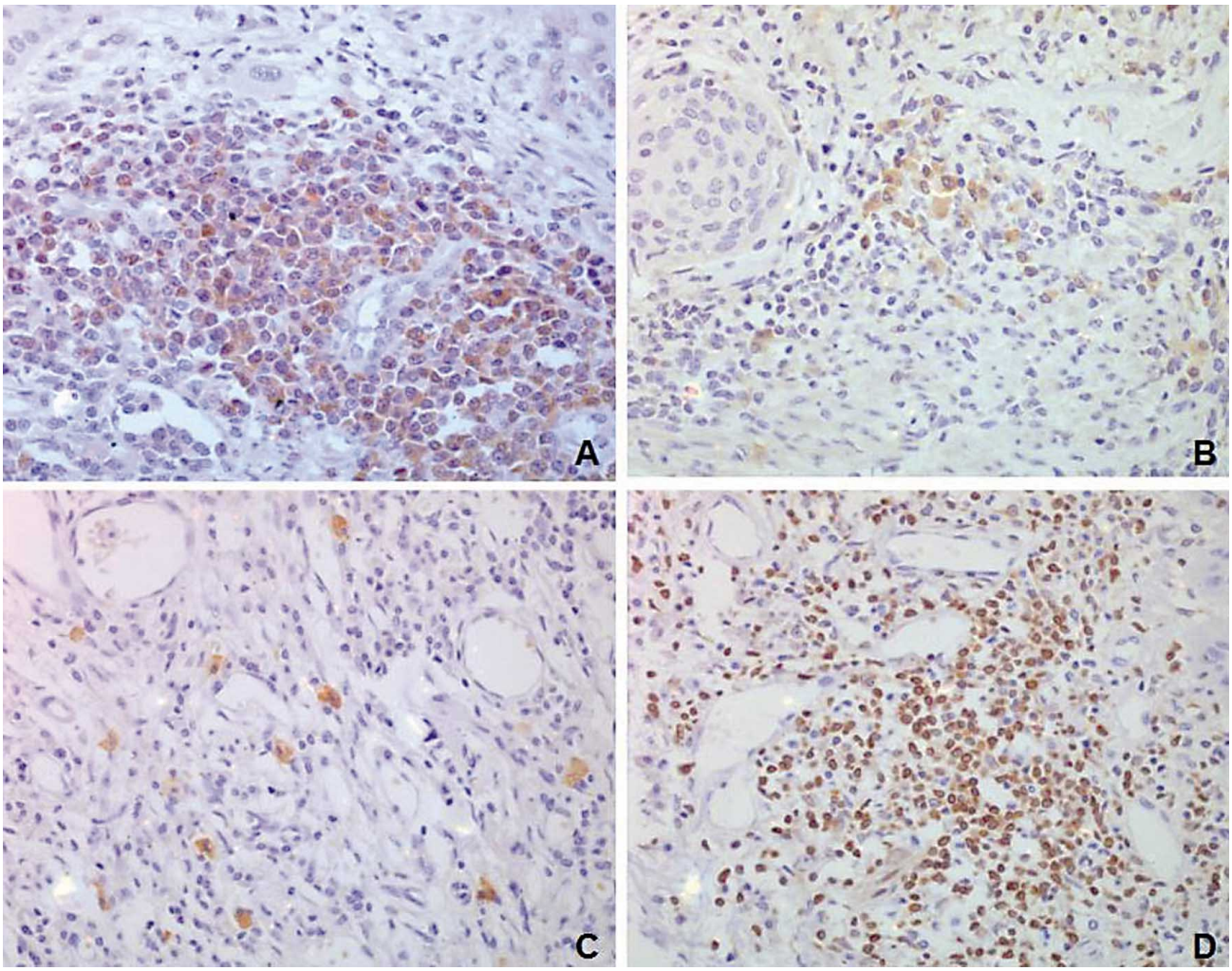

Fig. 1. Photomicrographs in epidermoid penile carcinoma. A- High caspase-3 expression (400x). B- Intermediate expression of caspase-3 (400x). C- Low expression of caspase-8 (400x). D- High bcl-2 expression (400x).

grade is showed in Fig. 2. It indicates that the expression of this protein is higher in patients with grade II. There was statistically significant difference between patients with grade I and II (p=0.0010, One-Way ANOVA, p<0.001, Postest of Tukey).

The intensity of bcl-2 expression was low in 28 cases (0 and +), intermediary in 11 cases (++) and high in 11 cases too (+++ and ++++) (Fig. 1D). It was observed that the expression of this protein was higher in patients with grade III. Patients with grade I and II showed a similar expression. No statistically significant difference was noted (p=0.1111, One-Way ANOVA test).

It was observed a low intensity of caspase-8 in all patients analyzed. A total of 49 cases presented low expression (+) and 1 case did not presented expression (0) (Fig. 1C). It was observed that the expression of this protein was higher in patients with grade I and its expression decreased as the tumor grade increased. However, no statistically significant difference was noted (p=0.5068, One-Way ANOVA test).

The relationship between the survival time of the patients, taking into account the clinical follow up and progression to death, and the tumor grade is indicated in Table II and Figure 3. It was observed statistically significant difference between the survival curves and the tumor grade (Logrank test and Qui-square, p=0.0351), only for grades I and II (p=0.0212).

The protein expressions were correlated with the survival time. Both proteins, caspase-3 and bcl-2, did not showed statistically significant difference after the use of Logrank test and the non-parametric test Qui-square (p=0.4346 and p=0.5119 respectively). This analysis was not applied to caspase-8 as all patients presented low expression of this antibody.

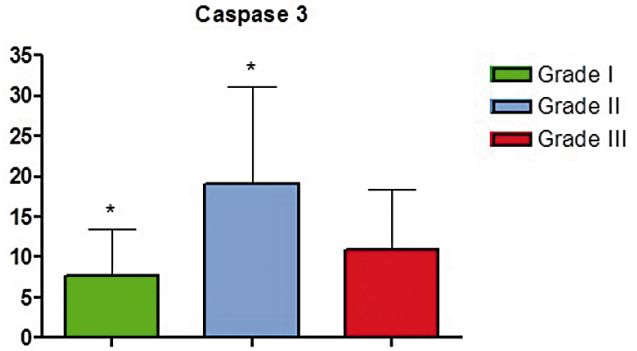

Fig. 2. Caspase-3 expression in the three tumoral grades, where *P < 0.05 is indicated with *. There were significant difference between tumor grades I and II (p = 0.0010, One-Way ANOVA test, p < 0.001 Pos-test of Tukey).

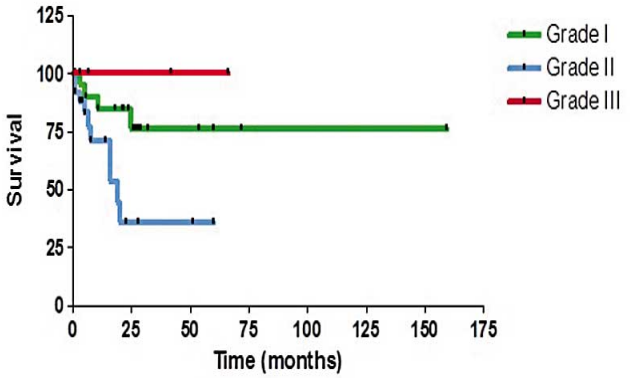

Fig. 3. Survival curves and patients follow (in months) and its correlation with the tumoral grade. There were statistically significant difference between the survival curves and the tumor grade (Logrank test and Qui-square, p=0.0351), only for grades I and II (p=0.0212).

Table II. Immunohistochemistry of caspase-3, caspase-8 and bcl-2 antibodies associated with the follow up time (months) and survival.

Tumoral grade	Graduation antibodies	Number of cases Caspase-3	Number of cases Caspase-8	Number of cases Bcl-2
I	Low	19	20	12
I	Intermediary	1	-	3
I	High	-	-	5
II	Low	18	26	16
II	Intermediary	8	-	6
II	High	-	-	4
III	Low	4	4	-
III	Intermediary	-	-	2
III	High	-	-	2

DISCUSSION

The apoptosis is an important mechanism that regulates cell growth. It is involved in a series of pathologies and influences the biological behavior of tumors. Its mechanism implicates a complex cascade of events divided into two pathways, which exhibit common stages. They are called intrinsic pathways and extrinsic pathway (Wyllie, 1995). The extrinsic pathway occurs due to ligand-specific activation of receptors from the super family of TNF receptor. In the intrinsic pathway the main event is the formation of apoptosome, which is a molecule, constituted of cytochrome c, APAF-1 and procaspase-9, that generate active caspase-9 which is responsible to cleavage and activate effectors caspases such as caspase-3 (Riedl & Shi, 2004).

Anomalies in the process of apoptosis can lead to an abnormal cellular growth and carcinogenesis. Among the cellular death regulators, there are the protein p53, bcl-2 and bax. The p53 protein represents a well-known apoptosis inductor and is the most common genetic mutation in cancer. The bcl-2 avoids apoptosis and has many important interactions, including its homology structure with bax that induce cellular death (Vasavada *et al*., 1998).

Martins *et al*. (2002) found that the immunohistochemistry expression of p53 in penile carcinoma can vary from 26% to 40% and exhibit correlation with Human Papiloma Virus (HPV). They analyzed the immunoreactions of p53, tumor stage (pT), grade, proliferating cell nuclear antigen (PCNA) as prognostic factor in 50 patients with squamous cell carcinoma (SCC) of penis, and compared with the, lymph nods onset and cause of death. The tumor stage, grade and PCNA expression do not showed significance for

lymph nodes metastasis and cause of death. However, the results showed that the immunoreactivity of p53 was the only factor with prognostic significance for the disease progression and the high expression was associated with lymph nodes metastasis, recurrence and cause of death. Therefore, it can be evaluated for therapeutic planning in this kind of tumor.

The pro-apoptotic protein bax and the anti-apoptotic protein bcl-2 are regulated by p53 (Saeed *et al*., 2005). Evidences have shown the p53 implication in the bax activation (Vasavada *et al*.). The apoptosis is regulated by bcl-2, depending on the relative concentration of its homo and heterodimers. The high concentration of bcl-2/bcl-2 and bax/bcl2 support cellular survival whiles the higher concentration of bax/bax lead to apoptosis. It is believed that pro-apoptotic factors in the mitochondria, such as cytochrome c, are released by bax, and promote caspases activation (Saeed *et al*.).

The expression of bcl-2 and bax are been proposed as prognostic factors in prostate cancer in patients treated with radiotherapy and surgery (Rubio *et al.,* 2005). The association between bcl-2 and prostate cancer is well known in immunohistochemistry analysis, showing an increase in the expression of this protein during prostate cancer progression (Coffey *et al.,* 2001).

Saeed *et al*., showed with immunohistochemistry technique that the expression of the protein bax was similar in verrucous carcinoma (VC) of penis and in well differentiate SCC. In addition, they observed that the expression of bcl-2 was statistically higher in well differentiate SCC.

Although no statistically significant difference was found in the analysis of bcl-2 in our study, we observed a higher expression of bcl-2 in patients with tumor grade III, which was expected. This increase in bcl-2 expression shows the role of this protein as an anti-apoptotic factor, as in Saeed et al., in penile carcinoma and in Coffey et al., in prostate cancer. The increase in bcl-2 expression related with bax in epidermoid carcinoma of penis lay emphasis in the importance of these proteins in the biologic feature of the lesion.

The caspase-3 can be considered the key executor of apoptosis. One immunohistochemistry study compared the prostatic benign hyperplasia (PBH) with prostate cancer (PCA) and demonstrated that PCA II and PCA III (Gleason system) have more apoptotic bodies than HPB and PCA I (Sohn *et al.,* 2000). The present study showed statistically significant difference for caspase-3 expression between tumor grades I and II and patients with grade I have expression values of this protein lower than patients grade II.

The caspase-8 is an initiator caspase that induce apoptosis in extrinsic pathway, but it can also change this pathway to the intrinsic one by BAD protein cleavage which releases mitochondrial proteins responsible for cellular death by activating caspase-3. Drewa *et al*., (2006) used immunohistochemistry analysis for FADD and caspase-8 in PCA, prostate intraepithelial neoplasia (PIN) and normal tissue (control), and observed that a high expression of these proteins, which are responsible for transmitting signals that induce apoptosis, appeared in PIN and PCA, and were negative in control. In this study, the immunoreactions of caspase-8 demonstrated that the higher the tumor grade, the lower the expression of this protein, with no statistically significant difference.

Our study showed statistically significant difference between the survival curves of tumor grade I and II. This result can be related with the great aggressiveness and large increase of tumor size in grade II. Furthermore, the expression of bcl-2 and caspase-3 were not able to change patient survival.

It is beyond doubt that studies related with epidermoid carcinoma of penis and its mechanism of apoptosis is scarce in the literature. For this reason, this immunohistochemistry study can contribute with information about this disease.

In this study, bcl-2 protein was more expressed than caspase-3 and caspase-8 proteins, suggesting that the apoptotic rate in this carcinoma is low. The higher expression of the anti-apoptotic protein bcl-2 suggests a higher preservation of the tumoral cells. Only grades I and II showed statistically significant difference when related with survival curves.

The analysis did not showed correlation between the proteins expression with the survival curve of patients.

NOVAIS, P. C.; DE PAULA, A. A. P.; REIS, R. B.; BARIONE, D. F.; FRANQUEIRO, A. G.; RODRIGUES JR., A. A.; LIZARTE, F. S. N.; DE CARVALHO, C. A .M.; TIRAPELLI, D. P. C.; PERIA, F. M.; SAGGIORO, F. P.; TUCCI JR., S.; COLOGNA, A. J.; MARTINS, A. C. P. & TIRAPELLI, L. F. Análisis inmunohistoquímico de proteínas relacionadas con la apoptosis como factor pronóstico en el carcinoma epidermoide de pene. ***Int. J. Morphol., 30(3)***:1029-1034, 2012.

RESUMEN: El objetivo fue analizar la expresión de las proteínas de genes de apoptosis caspasa-3, caspasa-8 y Bcl-2-con

la técnica de inmunohistoquímica, en correlación con el grado tumoral (I, II y III) y la supervivencia del paciente con el fin de comprender el mecanismo básico de la transformación tumoral. Se analizaron las reacciones inmunohistoquímicas sobre 50 muestras de carcinoma de células escamosas mediante el método de la inmunoperoxidasa avidina-biotina y la recuperación de antígeno. Los análisis se realizaron utilizando el método de graduación "en cruces" (0 a 4 cruces - no tinción a más del 75% de las células positivas) y en categorías (baja, media, alta) de la inmunorreactividad citoplasmática de las células de carcinoma epidermoide de pene. Se observó una diferencia estadísticamente significativa cuando la expresión de la caspasa-3 se comparó con los grados I y II del tumor (p = 0,0010) y cuando se comparan la supervivencia de los pacientes con los grados I y II del tumor (p = 0,0212). La proteína bcl-2 se expresa más que la caspasa-3 y caspasa-8, lo que sugiere que la tasa de apoptosis en este carcinoma es baja. La mayor expresión de la proteína anti-apoptótica bcl-2 sugiere una mayor preservación de las células tumorales.

PALABRAS CLAVE: bcl-2; Caspasa-3; Caspasa-8; Cáncer de pene; Apoptosis.

REFERENCES

Coffey, R. N.; Watson, R. W. & Fitzpatrick, J. M. Signaling for the caspases: their role in prostate cell apoptosis. *J. Urol., 165(1)*:5-14, 2001.

Drewa, T.; Wolski, Z.; Skok, Z.; Czajkowski, R. & Wisniewska, H. The FAS-related apoptosis signaling pathway in the prostate intraepithelial neoplasia and cancer lesions. *Acta Pol. Pharm., 63(4)*:311-5, 2006.

Guimarães, G. C.; Leal, M. L.; Campos, R. S.; Zequi, S. C.; da Fonseca, F. P.; da Cunha, I. W.; Soares, F. A. & Lopes, A. Do proliferating cell nuclear antigen and MIB-1/Ki-67 have prognostic value in penile squamous cell carcinoma? *Urology, 70(1)*:137-42, 2007.

Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell, 100(1)*:57-70, 2000.

Harden, S. V. & Tan, L. T. Treatment of localized carcinoma of the penis: a survey of current practice in the UK. *Clin. Oncol. (R. Coll. Radiol.), 13(4)*:284-7, 2001.

Martins, A. C.; Faria, S. M.; Cologna, A. J.; Suaid, H. J. & Tucci, S. Jr. Immunoexpression of p53 protein and proliferating cell nuclear antigen in penile carcinoma. *J. Urol., 167(1)*:89-93, 2002.

Mosconi, A. M.; Roila, F.; Gatta, G. & Theodore, C. Cancer of the penis. *Crit. Rev. Oncol. Hematol., 53(2)*:165-77, 2005.

Riedl, S. J. & Shi, Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat. Rev. Mol. Cell Biol., 5(11)*:897-907, 2004.

Rubio, J.; Ramos, D.; López-Guerrero, J. A.; Iborra, I.; Collado, A.; Solsona, E.; Almenar, S. & Llombart-Bosch, A. Immunohistochemical expression of Ki- 67 antigen, cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. *Eur. Urol., 48(5)*:745-51, 2005.

Saeed, S.; Keehn, C. A.; Khalil, F. K. & Morgan, M. B. Immunohistochemical expression of Bax and Bcl-2 in penile carcinoma. *Ann. Clin. Lab. Sci.,35(1)*:91-6, 2005.

Sohn, J. H.; Kim, D. H.; Choi, N. G.; Park, Y. E. & Ro, J. Y. Caspase-3/CPP32 immunoreactivity and its correlation with frequency of apoptotic bodies in human prostatic carcinomas and benign nodular hyperplasias. *Histopathology, 37(6)*:555-60, 2000.

Taylor, C. R.; Shi, S. R.; Chaiwun, B.; Young, L.; Imam, S. A. & Cote, R. J. Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. *Hum. Pathol., 25(3)*:263-70, 1994.

Vasavada, S. P.; Novick, A. C. & Williams, B. R. P53, bcl-2, and Bax expression in renal cell carcinoma. *Urology, 51(6)*:1057-61, 1998.

Wang, G.; Reed, E. & Li, Q. Q. Apoptosis in prostate cancer: progressive and therapeutic implications (Review). *Int. J. Mol. Med., 14(1)*:23-34, 2004.

Wyllie, A. H. The genetic regulation of apoptosis. *Curr. Opin. Gen et. Dev., 5(1)*:97-104, 1995.

Correspondence to:
Luis Fernando Tirapelli
Department of Surgery and Anatomy
University of São Paulo
Faculty of Medicine of Ribeirão Preto
Avenida Bandeirantes 3900
14049-900
Ribeirão Preto, SP
BRAZIL

Phone: +55-16-36024996

Email tirapeli@fmrp.usp.br

Received: 04-11-2011
Accepted: 28-05-2012