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Original Full Paper

Immunohistochemical analysis of E-cadherin and Caspase-3 expression in equine penile squamous cell carcinoma

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Abstract
Neoplasms are an important cause of morbidity and mortality in horses, with squamous cell carcinoma (SCC), also called squamous cell carcinoma, being the most common genital malignant tumor in the species and the most common neoplasm in horses in the State of São Paulo. Neoplasms frequently infiltrate the corpus cavernosum and can cause metastases in regional lymph nodes, generally having a guarded to poor prognosis due to local invasion and recurrence. Given the importance of the disease, the objective was to verify whether there is a difference in the expression pattern of immunomarkers of apoptosis and cell adhesion, according to the degree of cellular differentiation of the neoplasms. Twenty equine penile SCC samples from the Animal Pathology Service of the Veterinary Hospital of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo were histologically analyzed and classified according to their degree of differentiation. In addition, they were also subjected to the immunohistochemistry technique, with the immunomarkers Caspase-3 and E-cadherin. Data were analyzed using Kendall's correlation and the Mann-Whitney test. It was found that there is a positive correlation between the expression of immunomarkers and that there is no statistically significant difference in the expression of immunomarkers according to the degree of differentiation.

Keywords: apoptosis, cellular adhesion, neoplasm, penis.

Introduction

Neoplasms are an important cause of morbidity and mortality in horses, with squamous cell carcinoma (SCC), also called squamous cell carcinoma, being the most common genital malignant tumor in the species and the most common neoplasm in horses in the State of São Paulo. Both ultraviolet radiation and papillomaviruses have been reported in the pathogenesis of SCC in several animal species (3, 5, 12, 13).

At the beginning of the condition, it is possible to notice dermal hyperplasia and hyperkeratosis. As the condition progresses, there is erythema, edema, scaling, and possible ulceration. SCC grows rapidly, with a greater predilection for older animals. There is no consensus regarding its greater occurrence in stallions or castrated males, but many authors suggest that this neoplasm occurs more frequently in castrated animals, possibly due to the greater accumulation of smegma (15). Neoplasms frequently infiltrate the corpus cavernosum and can cause metastases in regional lymph nodes, generally having a guarded to poor prognosis due to local invasion and recurrence. The diagnosis is based on factors such as clinical symptoms and cytological examination, however, only the histopathological examination of the lesion is confirmatory (5, 16, 17).

It is believed that the inability to undergo apoptosis contributes to the evolution of carcinogenesis and the progression of neoplasms. It is known that ultraviolet light causes damage to the deoxyribonucleic acid (ADN) of epidermal cells, which can be repaired or eliminated through apoptosis, preventing carcinogenesis from occurring. Apoptosis

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markers can be marked using the immunohistochemistry technique, offering information that can be important and significant (7, 14).

Among the markers of apoptosis, we can mention Caspase-3, which is one of the main mediators of apoptosis, in addition to acting in its regulation. There is evidence of its participation in the progression of tumors and resistance to the treatment of various types of neoplasms, such as colorectal adenocarcinoma, breast carcinomas and cutaneous mast cell tumors, for example. (14).

In addition, there is also the important role of cadherins. They belong to a class of cell adhesion molecules, expressed on the surface of cells in all epidermal layers. E-cadherin is the main cadherin involved in epithelial cell adhesion and intracellular signaling that participates in mediating cell proliferation and motility (11). In the process of formation of carcinomas, the epithelial adherents junction is destabilized, characterized by decreased expression of E-cadherin.

The objective of this study was to verify whether the degree of cellular differentiation correlates with the expression of Casppase-3 and E-cadherin.

The hypothesis is that there is an inversely proportional relationship, where the greater the degree of differentiation, the lower the occurrence of apoptosis and cell adhesion (leading to metastases).

Material and Methods

Sampling

Samples of lesional tissue from 20 horses treated for treatment of histologically confirmed SCC were investigated. The samples were obtained from animals of different breeds (table 1) and included tumors of different degrees of differentiation (well differentiated and moderately differentiated). These samples came from the routine Animal Pathology Service of the Veterinary Hospital of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo. The study was authorized by the Ethics Committee on the Use of Animals of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo (CEUAx 5752230721).

Table 1. Breed of horses with penile squamous cell carcinoma treated at the Veterinary Hospital of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo.

Breed	Quantity
No defined breed	10
Mangalarga Marchador	5
Arabian	2
American Trotter	1
Margalarga Paulista	1
Campolina	1

Histopathological examination

SCC samples collected by surgical excision or punch for biopsies and preserved in paraffin blocks were used in 4-µm tissue sections to prepare slides that were stained with hematoxylin and eosin (HE) for routine histopathological examination. The degrees of differentiation considered the criteria established by the World Health Organization (WHO) (table 2).

Immunohistochemical analysis

Twenty samples of penile squamous cell carcinoma were subjected to immunohistochemical analysis. All immunohistochemical procedures were performed as previously described by João et al. (10). SCC slides were incubated for eighteen hours with rabbit polyclonal anti-Caspase-3 antibodies diluted 1:400 (code IM0035, company Rheabiotec, São Paulo, Campinas, Brazil) and anti-E-cadherin diluted 1:100 (code IM0066, company Rheabiotec, São Paulo, Campinas, Brazil), washed and incubated for one hour with SM802 EnVision ™ (code K4065, DakoCytomation, Santa Clara, USA). The chromogen DAB (3,3-diaminobenzidine – Dakocytomation code K3468-1) was used to reveal the reaction and counterstaining was done with Harris Hematoxylin and the slides were mounted with Entellan® (Merck, code HX888585). The brown color observed under optical microscopy indicated immunological reactivity. As a negative control, the primary antibody was excluded from the reaction, using only the antibody diluent (Dakocytomation, code S302283-2). For the positive control, neoplastic tissues (squamous cell carcinoma) were used.

To quantify the immunostaining in each section, the labeled cells were counted in five random fields, without 400X magnification (Nikon Eclipse E200 microscope). From the values obtained in these fields, an average number of immunostained epithelial cells per slide was calculated.

Table 2. Classification of squamous cell carcinoma suggested by the World Health Organization (WHO).

Classification	Characteristics
Poorly differentiated	Immature cells are the majority, many mitoses (atypical and typical), minimal keratinization.
Moderately differentiated	Nuclear pleomorphism, occurrence of mitosis, little keratinization.
Well differentiated	Very similar to normal squamous epithelium

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Statistic

The mean count of immunostained epithelial cells of the neoplasms was subjected to the Shapiro-Wilk test and data homoscedasticity test to verify whether they had a normal distribution pattern. The result showed that the distribution of the data was not normal, so Kendall's Correlation was performed (significance level $p<0.05$) to verify the difference between the expression of E-cadherin and Caspase-3.

To verify the difference in the expression of E-cadherin and Caspase-3 in SCCs according to their degree of differentiation, the non-parametric Mann-Whitney test was used (significance level $p<0.05$).

Results

Of the neoplasms analyzed, 65% were well differentiated (13/20) and 35% were moderately differentiated (7/20). There were horny pearls in 85% of the samples (17/20) and in 15% (3/20) were absent (figure 1). Necrosis and hemorrhage were present in 30% (6/20) of the samples and absent in 70% (14/20), and in 15% (3/20) both were present in the same sample. In the majority of samples, 60% (12/20), there was an ulcer, with, in two cases, many bacteria in the area. Mitotic figures were present in 85% of the samples (17/20) and absent in 15% (3/20), all cases of well-differentiated SCC. The inflammatory infiltrate was diffuse in 45% (9/20) of cases, multifocal in 45% (9/20) and focal in 10% (2/20). The intensity of expression of the immunomarkers varied according to the sample (Figures 2, 3 and 4).

Using Kendall's Correlation Coefficient, it was found that there is a positive and statistically significant correlation between the expression of E-cadherin and Caspase-3 in the study samples (0.68). No statistically significant correlation was found in the expression of E-cadherin ($p = 0.4497$) and Caspase-3 ($p = 0.7505$) according to the degree of differentiation of the neoplasms in the Mann-Whitney test.

Discussion

Regarding histological grade, 65% (13/20) were well differentiated and 35% (7/20) were moderately differentiated, none of the neoplasms were characterized in this study as poorly differentiated. In the study by Arthurs et al. (2020) (2), 39% (13/33) of cases were well-differentiated neoplasms, while 61% (20/33) were poorly differentiated. At the same time, Ramos et al. (20) obtained 55.56% of well-differentiated SCCs, corresponding to most of the sample, corroborating the present findings.

Most samples showed mitotic figures, regardless of the degree of SCC differentiation. Routine visual assessment of mitotic figures in histological sections is a gold standard method for evaluating proliferative activity and tumor grading (9). Dysregulated cell division is a central point in malignant neoplasms, as increased mitosis causes excessive cell proliferation, which can be observed in carcinomas (21).

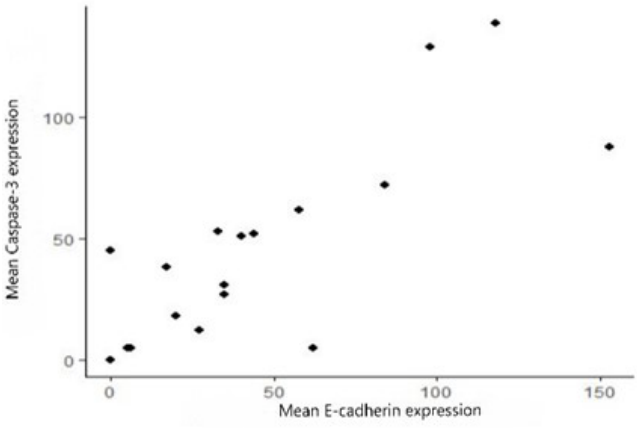

Figure 1. Scatterplot with the mean values of cells that were expressed.

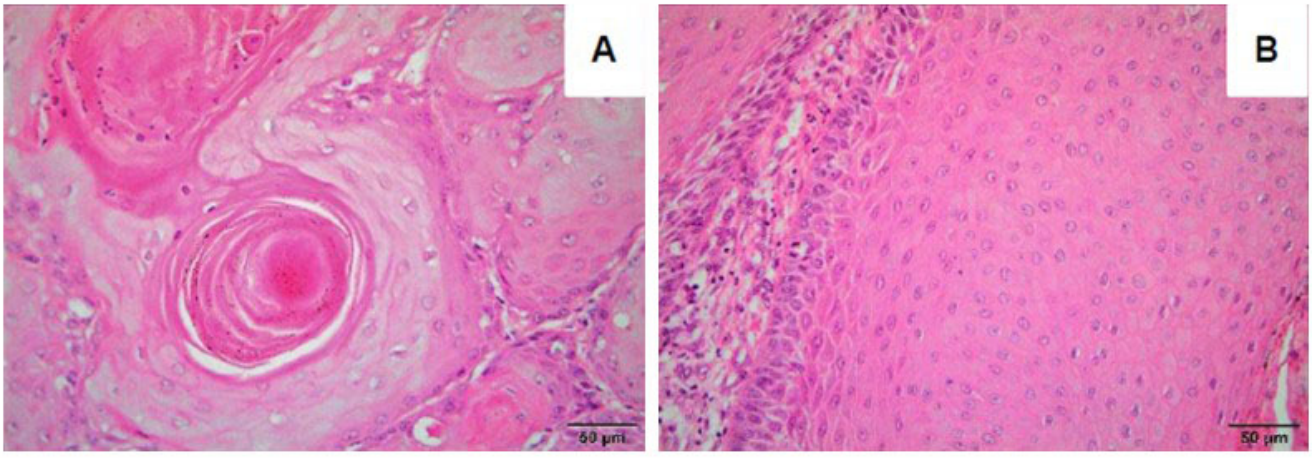

Figure 2. A- Keratin pearl in well-differentiated SCC (Hematoxylin and Eosin. Magnification 400X). B- Moderately differentiated SCC (Hematoxylin and Eosin. Magnification of 400X).

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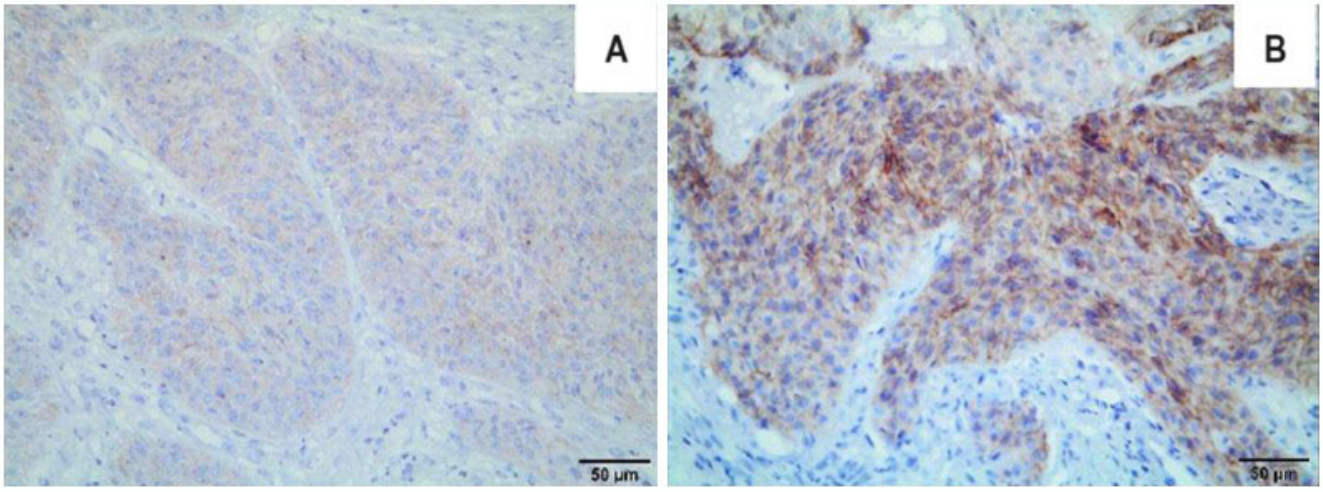

Figure 3. A- Weak positive immunostaining of E-cadherin in SCC moderately differentiated (400X magnification). B- Positive immunostaining of E-cadherin in SCC moderately differentiated (400x magnification).

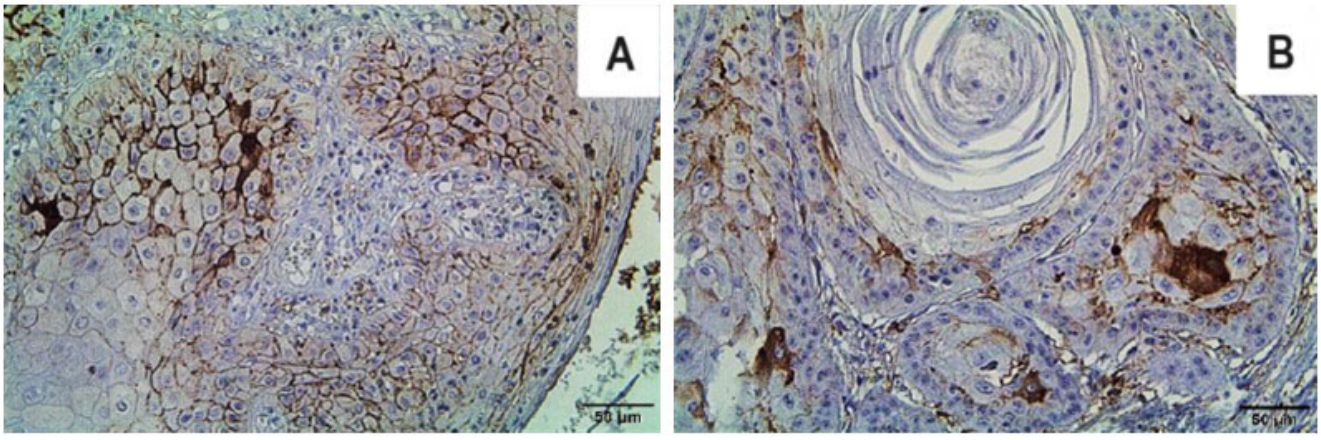

Figure 4. A- Positive immunostaining of Caspase-3 in well-differentiated SCC (400X magnification). B- Positive immunostaining of Caspase-3 close to the keratin pearl (400X magnification).

There were atypical mitoses in 5% (1/20) of cases, with SCC being moderately differentiated. Most tumors with atypical mitotic figures present some form of chromosomal instability and are frequently observed in highly proliferative and aggressive tumors. The identification of atypical mitoses is also of great value in distinguishing between benign and malignant tumors, along with other criteria (9).

Diffuse and multifocal inflammatory infiltrate was observed in 90% (18/20) of cases, with, in many cases, the presence of lymphocytes. Some studies highlight that the presence of lymphocytes may indicate a protective response or cytotoxic activity against neoplasia (22).

In 30% (6/20) of cases there was necrosis, 50% (3/6) in well-differentiated SCC and 50% (3/6) in moderately differentiated SCC. Necrosis is a common pathological change in squamous cell carcinoma, especially in large, poorly differentiated and rapidly growing tumors (11).

Tumor necrosis is commonly observed in central regions of tumors because of inadequate vascularization and subsequent metabolic stress such as hypoxia and nutrient deprivation. It is often associated with aggressive tumor development and metastasis, being considered an indication of poor prognosis (12). It is known that the expression of adhesion molecules, such as E-cadherin, is considerably lower in malignant neoplasms, when compared to normal tissues (1). This suggests that reduced E-cadherin expression may play an important role in invasion, metastasis and poor prognosis (6). In view of this, it is important to know if there is a difference in this expression, according to the degree of differentiation or malignancy of the same.

Caspase-3 expression is present in normal human tissues, overexpression and loss of Caspase-3 expression have been reported in several human malignancies (12). Huang et al. (8) analyzed 185 samples of squamous cell carcinoma of the oral mucosa in humans and found that

Caspase-3 expression was associated with an advanced stage of the neoplasia and a larger tumor size. Yang et al. (23) performed a meta-analysis of 3,091 cases to verify the association of Caspase-3 expression and clinical and pathological parameters in malignant breast neoplasms and no evidence showed that increased Caspase-3 expression was statistically correlated with the status of tumor differentiation (low/moderate or high) or lymph node metastasis.

Rare neoplasms in humans, such as penile SCC, have been largely neglected in urological research in favor of more common diseases. However, penile cancer represents a growing problem for health systems worldwide because the lack of knowledge, ineffective centralization of care and the absence of research funds make it difficult to improve global patient care (4).

Conflict of Interest

The authors declare no competing interests.

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