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Diagnostic and Prognostic Value of Cellular Proliferation Assessment with Ki-67 Protein in Dogs Suffering from Benign and Malignant Perianal Tumors*

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In the perianal region of carnivores, skin consists of modified sebaceous glands called perianal glands. Tumors originating from perianal glands are the third most frequent type of neoplasm in male dogs after neoplastic diseases of testes and skin. Ki-67 is a nuclear non-histone protein considered a proliferation marker in normal and neoplastic proliferating cells. Previous investigations revealed that Ki-67 expression may be used as a prognostic factor for breast cancer in humans. Thus, the aim of this study was to estimate the diagnostic and prognostic value of Ki-67 evaluation in dogs suffering from benign and malignant perianal tumors. The highest value of the Ki-67 index was obtained in the carcinoma group (18.50% ± 2.68), significantly higher compared to the values obtained in the control tissue (7.63% ± 2.12) and adenoma (7.33% ± 1.06; all $P<0.05$). Statistically significant differences in the Ki-67 index were not found between the epithelioma group (11.95% ± 1.96) and all other groups ($P<0.05$). This investigation on dogs with perianal gland tumors has shown significantly increased expression of Ki-67 antigen in carcinoma cells, while the expression of this protein was similar in the case of control tissues, adenoma and epithelioma. Thus, it may be postulated that Ki-67 evaluation in perianal gland tumors in dogs may serve as a useful marker possessing high diagnostic and prognostic value and enabling differentiation of malignant and benign tumors.

Key words: Dog, immunohistochemistry, Ki-67 protein, perianal tumors, proliferative activity.

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Tumors originating from perianal glands are the third most frequent type of neoplastic disease in male dogs, after neoplastic diseases of testes and skin (MORRIS & DOBSON 2001; ESPLIN *et al*. 2003; BURDZIŃSKA & IDZIAK 2008; MARTINS *et al*. 2008). Perianal glands are located in subcutaneous tissue, around the anal ring, at the base of tail, within the femoral region and on the prepuce. Histological evaluation has shown that the perianal gland does not have a secretive functions and its morphology is like that of hepatocytes, so tumors originating from these glands are called hepatoid adenoma, hepatoid adenocarcinoma or hepatoid carcinoma, depending on their malignancy (WILSON & HAYER 1979). The most frequent are adenomas constituting up to 80% of all cases. Malignant adenocarcinomas may be followed by systemic symptoms such as apathy, muscle weakness, polyuria and polidypsia associated with mineral metabolism disturbances, hypercalcemia and hypophosphatemia (BERROCAL *et al*. 1989; POLTON & BREARLEY 2007). The cellular surface and nucleus of perianal glands are characterized by the presence of androgen or estrogen receptors and tumor growth may

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be stimulated by sex hormones (DE LAS MULAS *et al*. 2000; MILLANTA *et al*. 2005; PISANI *et al*. 2006). Perianal glands cells show sexual dimorphism due to regression of the glands in mature females and glandular masses present in males (SHABADASH & ZELIKINA 1995). In clinical examination, perianal gland tumors appear as nodular formations with a tendency to bleed on reaching a considerable size. Metastases from primary tumor may occur in lungs, liver, spleen, as well as iliac and lumbar nodes (MARTINS *et al*. 2008). Among the various features of neoplastic process development such as acceleration of amino acid metabolism and influences within macro- and microelement turnover, characteristic changes on cellular level are related to DNA replication (BRODZKI *et al*. 2004a, b; BRODZKI *et al*. 2005; BRODZKI *et al*. 2013a, b). In proliferating normal and neoplastic cells, increased DNA synthesis is associated with an expression of nuclear proteins that may function as biological process markers within several types of tissue. Experimental studies in humans and animals have shown significant differences in expression of proliferating cell nuclear antigen (PCNA) and Ki-67 antigen during the course of malignant and non-malignant neoplastic cell proliferation processes (ZHONG *et al*. 2008; ŁOPUSZYŃSKI *et al*. 2009; PEREIRA *et al*. 2013). Thus, the aim of the study was to evaluate the diagnostic and prognostic value of Ki-67 antigen assessment in dogs suffering from perianal gland tumors and its potential ability to differentiate between malignant and non-malignant neoplastic processes.

Material and Methods

The experimental procedures used throughout this study were approved by The II Local Ethics Committee on Animal Experimentation of University of Life Sciences in Lublin, Poland – reference number 49/2006.

This investigation was performed on 41 tissue samples obtained as the result of perianal gland tumor biopsy with the use of a 6 mm trepan. The samples were collected from dogs treated in the Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland. Control tissue samples containing normal perianal glands were also collected from six healthy dogs. All tissue samples were fixed in 10% neutral formalin pH 7.2 for the duration of 24 hours. Serial sections were cut at 4 μm using a sledge microtome, stained with hematoxylin and eosin and closed with the use of DPX (Sigma-Aldrich). Histopathological analysis was performed using a light microscope Nikon Eclipse E-600 in accordance with AFIP/WHO classification (GOLDSCHMIDT *et al*. 1998). Tissue sections intended for use in immunohistochemical evaluation were transferred onto silane coated glass slides Super Frost (Menzel-Glaser) and left at 56°C for 12 hours. Prior to immunohistochemical reaction of antibody and Ki-67 antigen, the preparations were transferred to Antigen Unmasking Solution (Vector), at a dilution ratio of 1:100 and unmasked in a decloaking chamber for 8 min. Following this procedure, they were left in the chamber for 60 min, and left at room temperature for 20 min following removal from the chamber. Ki-67 antigen expression was assessed using Dako-ARK^{TM} set for the immunohistochemical staining of animal tissues (Animal Research Kit, Dako) reducing the background of non-specific reactions. Mouse monoclonal antibody (clone MIB-1) against Ki-67 protein was used (Dako). This antibody is intended for processing of paraffin-embedded tissues at 1:300 dilutions. In order to obtain a color reaction, the preparations were incubated with 3,3'-diaminobenzidine tetrahydrochloride solution (DAB) used as a chromogen. A double control system was applied for the immunohistochemical reactions. For negative controls, incubation with primary antibodies was replaced by incubation with a relevant quantity of mouse IgG serum, while the positive control comprised healthy tissue of canine tonsils showing a positive reaction to the antibody. Quantitative determination of Ki-67 antigen expression, as the result of immunohistochemical reactions, was performed using a computer aided microscopic image analysis system. In this system, a light microscope (Nikon Eclipse E-600) was linked with a digital camera (Nikon DS-Fi1) and PC unit equipped with image analysis software (NIS-Elements BR-2.20, Laboratory Imaging). The computer-aided microscopic image analysis system was used for data storing as well. Ki-67 expression was determined at a lens magnification of x 40, and the index value was calculated as the percentage of immunopositive cells per 500 neoplastic cells.

Statistical analysis was performed using Statistica software (version 6.0) and one-way analysis of variance (ANOVA). Post-hoc Tukey's HSD test was used to compare differences between the investigated groups. The differences between mean values were considered as statistically significant at $P<0.05$.

Results

Histopathological evaluation of perianal gland tumors revealed 24 cases (58.5%) of adenoma, 12 cases (29.3%) of epithelioma and 5 cases (12.2%) of carcinoma (Table 1). Positive immunohistochemical reaction confirming the presence of the

Table 1

Histopathological classification of perianal gland tumors and their incidence in dogs

Histological classification	No.	Percentage incidence
Adenoma	24	58.5%
Epithelioma	12	29.3%
Carcinoma	5	12.2%
Total	41	100.0%

Ki-67 antigen was observed in cellular nuclei, excluding a rarely observed weak intensity reaction in the cytoplasm of some epithelial cells, therein cells showing mitotic fragmentation figures. The observed reaction was granular or granularly-fuzzy. Very intensive positive staining was often found in the case of the nucleolus. The differences in staining intensity between particular nuclei were slight. The distribution of the Ki-67 antigen positive cells was characteristically heterogeneous. In the case of normal perianal gland tissue sections and adenoma, the cells showing positive immunostaining reaction were located in the peripheral zone of trabeculae, islands or cords in a group of basaloid reserve cells. In case of hepatoid adenomas, only a few cells showed a positive reaction for the presence of the Ki-67 antigen. Similar observations were made in the epithelioma group in which the majority of the positive immunostaining reaction was found in the basaloid reserve cells at the border of the structures forming the tumor tissue. However, hepatoid cells with an intensively immunostained nucleus were also observed in this group. In the carcinoma group, poorly differentiated positive cells were disseminated chaotically among negative cells. Immunostained nuclei in the stromal cells and vascular epithelium were observed occasionally. All epithelial cells showing intensive immunostaining within their nuclei were considered as displaying a positive reaction (Figs 1-3).

The results showing Ki-67 antigen expression within several tissues are shown in Fig. 4. Ki-67 index values in all the investigated tissues were between 2.20% and 25.40%. The highest value of the Ki-67 index was obtained in the carcinoma group (18.50% ± 2.68), and it was significantly higher than the values for control tissue (7.63% ± 2.12) and adenoma (7.33% ± 1.06; all $P<0.05$). Statistically significant differences in Ki-67 index values were not found between the epithelioma group (11.95% ± 1.96) and all the other groups ($P>0.05$).

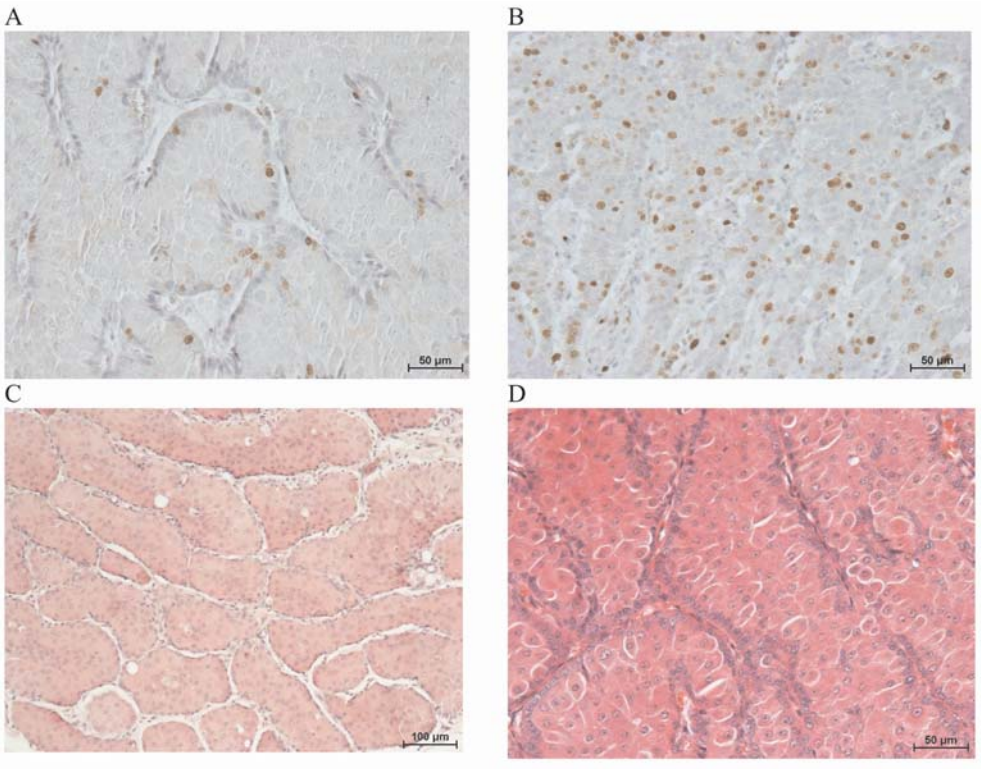

Fig. 1. Ki-67 antigen expression in perianal gland adenomas. A – Few of the basaloid reserve cells showing a positive immunostaining reaction (×200). Bar = 50 μm. B – Positive immunostaining reaction in basaloid and hepatoid cells. Immunohistochemical staining for Ki-67 antigen (×200). Bar = 50μm. C – Adenoma of the perianal glands. Hematoxylin and eosine staining (×100). Bar = 100 μm. D – Adenoma of the perianal glands. Hematoxylin and eosine staining (×200). Bar =50 μm.

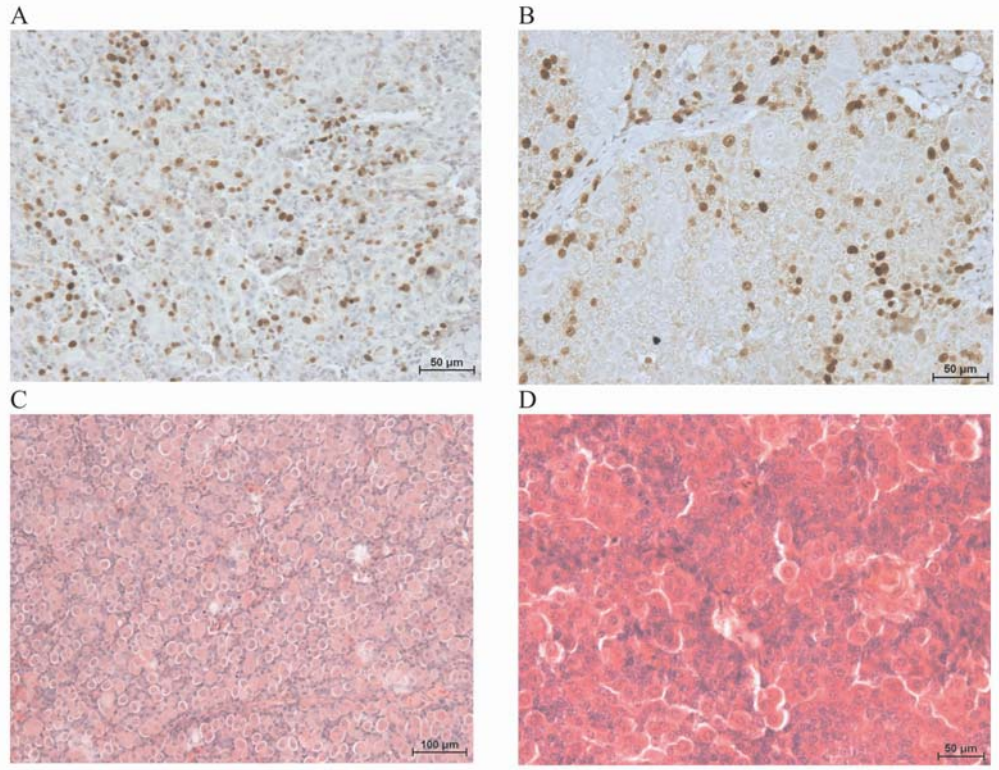

Fig. 2. The differences in staining intensity for the presence of Ki-67 antigen in perianal gland epitheliomas. A – Ki-67 antigen expression dominating in the basaloid reserve cells (×200). Bar = 50 μm. B – moderate and strong immunostaining reaction showing Ki-67 antigen expression in basaloid and hepatoid cells. Immunohistochemical staining for Ki-67 antigen (×200). Bar = 50 μm. C – Proliferating basaloid cells of epithelioma of the perianal glands. Hematoxylin and eosine staining (×100). Bar = 100 μm. D – Proliferating basaloid cells of epithelioma of the perianal glands. Hematoxylin and eosine staining (×200). Bar = 50 μm.

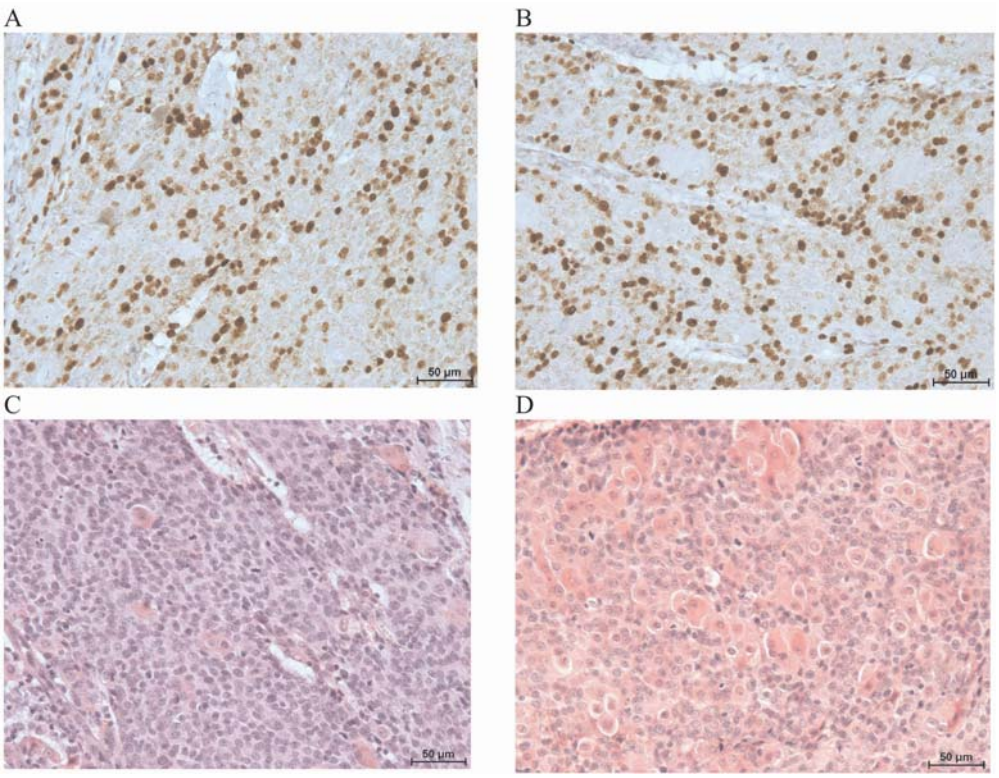

Fig. 3. Increased expression of Ki-67 antigen in perianal gland carcinomas. A – Numerous cell nuclei exhibiting a positive reaction to Ki-67 antigen. Immunohistochemical staining for Ki-67 antigen (×200). Bar = 50 μm. B – Strong positive reaction to Ki-67 antigen predominantly present in poorly differentiated basaloid reserve cell population. Immunohistochemical staining for Ki-67 antigen (×200). Bar = 50 μm. C – Carcinoma of the perianal glands with single hepatoid cells placed nearly non-differentiated basaloid cells with numerous mitotic figures. Hematoxylin and eosine staining (×200). Bar = 50 μm. D – Carcinoma of the perianal glands with single hepatoid cells placed nearly non-differentiated basaloid cells with numerous mitotic figures. Hematoxylin and eosine staining (×200). Bar = 50 μm.

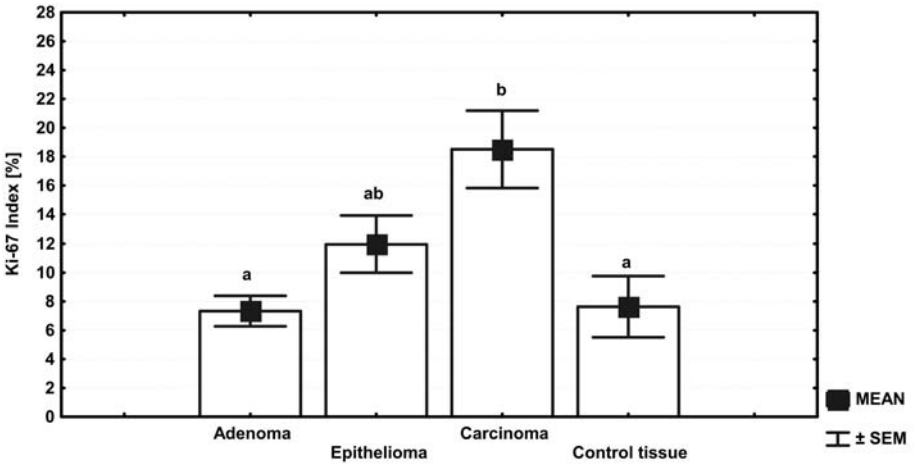

Fig. 4. Percentage of Ki-67 index showing antigen expression in control tissue, and in adenomas, epitheliomas and carcinomas of perianal glands. Statistically significant differences between groups are marked with different letters for $P<0.05$.

Discussion

Due to increasing incidence of neoplastic cases in veterinary practice, animal oncology requires novel and valuable assays and techniques to identify diagnostic and prognostic markers of neoplastic diseases and their introduction to standard histopathological evaluation. Different etiology and metabolic processes during neoplastic growth in various tumor types in dogs require individual recognition of diagnostic and prognostic values of several neoplastic markers (MORRIS & DOBSON 2001). A lack of experimental studies in dogs examining the usefulness of immunohistochemical methods for diagnostic and prognostic purposes of tumors originating from the perianal glands was pivotal for the decision to perform the current study.

The etiology of perianal gland tumors in dogs is unknown but the growth of neoplastic cells is modulated by sex hormones such as androgens and estrogens (HAYES & WILSON 1977). Regardless of perianal tumor etiology, neoplastic growth is characterized by changes appearing at the tissue level such as cellular proliferation and apoptosis. Proliferating cell nuclear antigen (PCNA) is an acidic nuclear protein involved in eukaryotic DNA synthesis and plays an important role in the regulation of the cell cycle. Proliferating cell nuclear antigen occurs during DNA replication in both normal and transformed tissues. PCNA expression is significantly up-regulated in the process of malignant transformation of normal epithelium and is considered a marker reflecting cell proliferation activity in most tumor types (MARTINS *et al*. 2008; MENG *et al*. 2013). The other proliferation marker present in all proliferating cells is Ki-67 protein (GERDES *et al*. 1983). The antibody against Ki-67 reacts with 395 kDa nuclear non-histone protein that is present in all active phases of the cell cycle, except the G0 phase (CATTORETTI *et al*. 1982). In human medicine, Ki-67 is one of the 21 prospectively selected genes used to predict the risk of recurrence and the extent of chemotherapy benefits in women with node-negative and ER-positive breast cancer (PAIK *et al*. 2004; PAIK *et al*. 2006). Thus, the proliferation marker Ki-67 is considered a prognostic factor for breast cancer in humans (NISHIMURA *et al*. 2010). Considering these data, the estimation of diagnostic value of Ki-67 evaluation in perianal gland tumors in dogs was performed in this study.

Adenoma was most prevalent in our study. This type of tumor reached nearly 60% incidence among dogs suffering from neoplastic changes of perianal tumors. These neoplastic changes were characterized by relatively low proliferative activity as expressed by Ki-67 protein evaluation, comparable to that of control dogs. Epithelioma was the second most prevalent neoplasm of the perianal glands reaching 30% in the investigated group of animals. The proliferative activity as shown by Ki-67 expression was relatively poor and comparable to the expression of this marker in adenoma or control tissue. In contrast to dogs with adenoma and epithelioma, the incidence of carcinoma was the lowest; however, cellular expression of Ki-67 and cellular proliferative activity was the highest in this group. The obtained results are in agreement with studies on dogs performed by MARTINS *et al*. (2008) where cell proliferation was quantified by evaluation of PCNA in cellular nuclei in different histological types of neoplasia of these glands. In this study, the incidence of adenoma, epithelioma and carcinoma was similar to our observations and reached 54%, 19% and 20%, respectively. Analogically to Ki-67 expression in our study, quantification of positive nuclei for

PCNA in adenomas was not significantly different to control tissue. In contrast to the results of Ki-67 expression obtained in the current study, the previous report showed a significantly increased amount of PCNA positive nuclei in epithelioma cells by 213% when compared to the normal tissue. However, in both these studies evaluation of Ki-67 and PCNA revealed significant differences between dogs suffering from carcinoma of perianal glands and the controls. In the current study, the expression of Ki-67 in carcinoma cells increased by 142% when compared to the normal cells, while PCNA evaluation in the previous studies showed a nearly 500% difference between these cells (MARTINS *et al*. 2008). Considering the data from both studies, it may be postulated that Ki-67 evaluation in perianal gland tumors has diagnostic and prognostic value to differentiate carcinoma (malignant neoplastic process) from adenoma, which is deemed a benign neoplastic change. The higher PCNA index values in comparison to the Ki-67 index values can be explained by the relatively long half-time of PCNA protein in cells. It is assumed to last for about 8 hours for dividing cells, and about 20 hours for cells which have passed the resting phase (MORRIS & MATHEWS 1989). Another explanation of high PCNA index values may be its participation in numerous cell metabolic processes, including DNA repair, occurring with increased activity in neoplastic tissues (MAGA & HUBSCHER 2003). The most problematic interpretation of immunohistochemical examination seems to be the case of epithelioma, since Ki-67 antigen expression was not different from all other groups. In the case of PCNA evaluation in perianal gland tumor cells, the results obtained by MARTINS *et al*. (2008) showed a corresponding increase of PCNA positive cells in dogs with epithelioma and carcinoma when compared to cells of control tissues or adenoma. Furthermore, identical findings and conclusions were obtained evaluating apoptotic corpuscles in these dogs (MARTINS *et al*. 2008). The results obtained in our study are in agreement with studies on rats with preneoplastic and neoplastic stages of hepatocarcinoma in which immunohistochemistry for PCNA was shown as an additional tool to differentiate benign and malignant tumors (KONG & RINGER 1996). The other report confirms the usefulness of immunohistochemical evaluation of PCNA expression to differentiate malignant and benign neoplasms and its correlation with prognosis. Unlike our findings in dogs, these conclusions were based on a study on women with benign and malignant epithelial ovarian neoplasms. The fact that PCNA was found in 6% of patients possessing benign neoplastic changes, while positive nuclear staining for PCNA was stated in only 60% of women suffering from malignant epithelial ovarian neoplasms, is also noteworthy (NAKOPOULOU *et al*. 1993). In studies on 131 bitches suffering from malignant mammary tumors, the Ki-67 antigen index value achieved a statistically higher value in dogs with neoplastic disease progression when compared to animals without recurrence and metastases. The Ki-67 antigen index value attained better precision for predicting the remission time and survival time than the PCNA index. Considering the highly diagnostic and prognostic value in dogs with malignant mammary tumors, the evaluation of Ki-67 was postulated for introduction into standard histopathological examination (ŁOPUSZYŃSKI *et al*. 2009). The diagnostic and prognostic value of Ki-67 evaluation in canine perianal gland neoplasms was also confirmed in studies on male and female dogs. Immunostaining for Ki-67 revealed that the carcinomas showed a higher proliferation rate (9.87%) than in the groups with epitheliomas (2.66%) and adenomas (0.36%). Moreover, higher Ki-67 index was found to be related to recurrence in cases of perianal gland carcinomas (PEREIRA *et al*. 2013). Significantly higher values of the Ki-67 index were also found in bitches showing clinical symptoms of a malignant disease such as mammary gland inflammation or the presence of metastases when compared to healthy controls (GIZIŃSKI *et al*. 2003). In studies on humans, Ki-67 was shown to be a prognostic factor for gastrointestinal stromal tumor risk of recurrence or spread of neoplastic disease (BELEV *et al*. 2013). Another cohort study on 3652 patients revealed in multivariate analysis that the Ki-67 index value was a significant factor for determination of disease-free survival and overall survival rates in women with primary breast cancer. Ki-67 index increased by 20% or higher and significantly correlated with other proliferation markers, poorer prognosis and early recurrence, particularly in luminal A type tumors. Moreover, a higher Ki-67 index value was significantly correlated with a higher grade of tumor malignancy in these patients (NISHIMURA *et al*. 2010). In a group of 140 men with prostate cancer, higher expression of Ki-67 value was associated with increased proliferation rate of tumor cells and postulated as a valuable marker of biological tumor aggression, selecting patients for more or less aggressive treatment (LUCZYNSKA *et al*. 2012).

In conclusion, our investigations on dogs with perianal gland tumors have shown significantly increased expression of Ki-67 antigen in carcinoma cells, while the expression of this protein was similar in the case of control tissues, adenoma and epithelioma. Thus, it may be postulated that Ki-67 evaluation in perianal gland tumors in dogs can serve as a useful marker possessing highly diagnostic and prognostic value and enabling differentiation of malignant and benign tumors.

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