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Correlation between oestrogen receptor (ERα) and Ki-67 receptor expression in canine genital neoplasms by immunohistochemistry#

Kodange Omkar Tukaram[1], Shibu Simon[2*], K. Magnus Paul[3], C. Jayakumar[4], I. S. Sajitha[5] and T. Vanesa John[6]

Department of Animal Reproduction, Gynaecology and Obstetrics,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651,
Kerala Veterinary and Animal Sciences University, Kerala, India.

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Abstract

The objective of the current study involved assessment of correlation between the expression of oestrogen receptor (ERα) and Ki-67 by immunohistochemistry, in the genital borne neoplasms in 12 postpubertal bitches between 2 to 12 years of age, presented at University Veterinary Hospitals, Kokkalai and Mannuthy. The age group of 5-8 years had the highest incidence of neoplasia (50.00 %) in comparison with other groups. Episiotomy was performed for extraluminal tumours while local excision performed in all intraluminal tumours except canine transmissible venereal tumour (CTVT). A chemotherapeutic regimen involving intravenous infusion of vincristine sulphate at 0.025 mg/kg was administered to bitches with CTVT and squamous cell carcinoma (SCC) until complete remission of the neoplasm. The tumours were histopathologically confirmed as myofibroma (n=6), CTVT (n= 3), leiomyoma (n = 1), SCC (n = 1) and fibroma (n = 1). Immunohistochemical staining of the neoplastic tissues was performed to evaluate ERα and Ki-67 expression status which could aid in estimating the malignancy potential and influence of ERα on the same. Spearman's rank correlation between the immunohistochemical scores for the expression of ERα and Ki-67 in these tumours was highly significant ($p<0.01$). The correlation between ERα and Ki-67 had a significant negative correlation ($p < 0.05$). In conclusion, the study suggests that higher expression of Ki-67 of a neoplasm coupled with lower expression of ERα bears evidence to a malignant process.

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1. *M.V.Sc. Scholar*
2. *Associate Professor*
3. *Assistant Professor*
4. *Associate Professor and Head*
5. *Assistant Professor, Department of Veterinary Pathology*
6. *Assistant Professor, Department of Pathology, JMMCRI, Thrissur*

**Corresponding author: shibu@kvasu.ac.in, Ph: 9447006499*

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Cancer-related conditions have been impacting the quality of life in canines. Cancer of the reproductive organs is presented in a clinically subtle manner, rendering clinical diagnosis tricky. Steroid hormones such as oestrogen, progesterone and androgen probably have a significant role in the growth of reproductive tumours, including mammary tumour (Millan *et al*., 2013), ovarian tumour and leiomyoma (Agnew and Maclachlan, 2016). The rate of incidence of genital neoplasia is sparse owing to the prophylactic practices like early neutering of the canines. However, intact geriatric bitches or those exposed to surgical mishaps like incomplete resection of ovarian tissue during ovariohysterectomy (OHE) remain vulnerable to reproductive tumours. The growing demand of canines for companionship establishes the need for newer advancements and standardisation of oncological surgical procedures for canine patients. The designing of target selective therapies for hormonally influenced tumour and grading their prognosis can be optimised by employing molecular grading for cancer. Immunohistochemical staining with specifically labelled antibodies for visualisation of target protein within the tissue sections extracted from tumours is a proven efficient molecular grading method for tumour characterisation. The current study was designed to evaluate the immunohistochemical expression of oestrogen receptor alpha (ERα) and Ki-67 in the genital tumours. Ki-67, better known as proliferation marker, is a molecular tool with standardized efficacy as a marker for high grade or malignant tumours.

Materials and methods

Selection of animals

The current study was conducted in 12 post-pubertal bitches between the age group of 2 to 12 years with symptoms suggestive of genital neoplasia like stranguria, aberrations of oestrous cycle, haemorrhagic vaginal discharge, faecal tenesmus, perineal swelling, visible protruding mass and inappetence. Clinico-gynaecological examination was performed for recording their vital physiological parameters comprising of pulse rate (beats per minute), respiratory rate (breaths per minute), rectal temperature (°C), colour of visible mucus membrane and status of superficial lymph nodes. Vulva, vestibule and vagina were explored to detect presence of tumours. Mammary glands were checked for neoplastic lesions. Physical examination of the external genitalia with gloved hand was performed to note the location of tumour mass. Per vaginal examination was performed with a sigmoidoscope to visualise the neoplastic growth.

Exfoliative vaginal cytology and vaginoscopy

Exfoliative vaginal cytology was performed to detect the presence of exfoliated cells and neoplastic cells in vagina, if any. The cells were procured from the selected animals on the day of presentation by introducing sterile cotton tipped swab into the caudal vagina as suggested by Simon (1997). A fibre optic Sigmoidoscope (WelchAllyn®, Skaneateles Falls, NewYork) was passed through vulvar opening and directed towards anterior vagina to evaluate the presence of neoplastic lesions of vaginal tract, if any.

Haematological parameters

Blood was collected from cephalic vein for complete blood count and serum sample for evaluating blood urea nitrogen (BUN), creatinine and alkaline phosphatase (ALP) on the day of presentation.

B-mode ultrasonography

Ultrasonographic examination of uterus and ovaries was done to detect the presence of any uterine or ovarian pathology using a transducer with frequency of 5-7.5 MHz (Sridevi, 2013). The visceral organs like kidneys, liver and spleen were screened to evaluate metastasis if any. Transabdominal approach was done with a multifrequency Doppler ultrasonography. The colour Doppler was utilized to visualize the vessels and pulse wave (PW) Doppler was utilised to measure the perfusion to the tumours. The resistivity index (RI) and pulsatility index (PI) of the blood vessel supplying the tumour was recorded.

Doppler ultrasonography

The colour Doppler was utilized to visualize the vessels and pulse wave (PW) Doppler was utilised to measure the perfusion to the tumours. The resistivity index (RI) and pulsatility index (PI) of the blood vessel supplying the tumour was recorded (Simon *et al.,* 2021).

Radiography

The animals were subjected to radiographic evaluation of abdomen and thorax to detect the presence of metastasis. The animals were placed in right lateral recumbency. Thorax and abdomen were radiographed separately. The abdomen was screened for any metastatic lesions and displacement of the viscera by the tumour (Russo *et al*., 2021).

Collection of material

The incisional biopsy technique was implemented for collection of the tumour tissue from animals presented with friable tumours consisting of ulcerated surfaces which had tendency to bleed easily, like CTVT. The tissues from the indwelling tumours of tubular genitalia or vulva were taken following excisional biopsy. The collected sample was fixed in neutral buffered formalin (NBF) for 48 hours and subjected to histopathological and immunohistochemical evaluation (Christy *et al*., 2022).

Histopathological evaluation

Tissues were processed for histopathology. Tissue sections of 5µm thickness were prepared and stained by haematoxylin and eosin technique (H&E) (Bancroft and Gamble, 2008). The slides were examined to determine the type of tumour. Histopathological characteristics of the tumours were recorded using Magcam DC5 software.

Immunohistochemical evaluation

The sections procured from the tumours were subjected to immunohistochemical staining. These samples were then scored according to the scoring pattern advised by Vakkala *et al*. (1999). The correlation of these scores was performed using Spearman's rank correlation using SPSS 24.

Results and discussion

Selection of animals with genital neoplasia for the study

Out of the 12 bitches selected, six were in the age group of 5-8 years, five were in the age group of 1-4 years and one was in the age group of 9-12 years. This was similar to the findings of Brodey and Roszel (1967) and Lee *et al*. (2014). The mean age of occurrence of genital tumours in canines in the current study was 5.90 ± 0.98 years, although Kydd and Bernie (1986) reported the mean age to be 11.2 years. The difference in the mean age group may be due to early detection of neoplasms in the canines under current study.

The clinical signs on the day of the presentation included stranguria (58.30 %), aberrations of oestrous cycle (16.60 %), haemorrhagic vaginal discharge (91.60 %), faecal tenesmus (50.00 %), perineal swelling (33.30 %), protruding mass (66.60%) and inappetence (58.60 %). The clinical signs enlisted were in accordance with Kang and Holmber (1983), Kydd and Bernie (1986), Stockmann *et al.* (2011) and Sahoo *et al.* (2020). The observation of intraluminal masses in vestibule/vagina and the perineal swelling in the extraluminal masses was in agreement with the findings of Umamageswari *et al*. (2016).

The lymph nodes were enlarged in 25.00 per cent of the animals. No inflammation or neoplastic mammary glands were observed in any of the animals under the study. The neoplasms were distributed in anterior vestibule (42.00 %), vestibulo-vaginal junction (33.00 %) and on vulva (25.00%). Canines affected with CTVT were 25.00 percent, represented as ulcerated mass in vestibule or vulva. These findings were similar to the reports by Amaral *et al*. (2007) and Ganguly *et al.* (2016). Ganguly *et al.* (2016) reported that percentage of distribution of neoplasms along vestibule, vagina and vulval lips as 95.60, 44.50 and 18.60 per cent, respectively. Leiomyoma (8.30 %) was observed as an intraluminal mass

suspended by a stalk, attached to the vestibulo-vaginal junction. The growth of the neoplasm in that location might be due to the mesenchymal component present in the tubular genitalia. The myofibromas were present extraluminally as a perineal swelling attached to dorsal aspect of vaginal wall.

Exfoliative vaginal cytology and vaginoscopy

Exfoliative vaginal cytology of canines under the present study was suggestive of dioestrus and anoestrus stage of oestrous cycle in 25.00 and 75.00 per cent of affected canines, respectively. The appearance of the exfoliated vaginal cells was similar to the descriptions of Simon (1997), Jhonston *et al*. (2001) and Kustritz (2020). As a common feature, those animals presented with CTVT were in dioestrus stage on cytological evaluation. The appearance of CTVT cells was similar to the description by Nair (2012) and Priyadarshani *et al*. (2021).

Haematological parameters

The mean values of all haematological parameters derived from whole blood and serum were within the normal reference range with the exception of alkaline phosphatase (Table 1). The results of current study were comparable to the results of Lee *et al.* (2014). The mean values of alkaline phosphatase were slightly elevated in comparison to normal physiological range (21-212 IU), this elevation might be related to neoplasia as suggested by Karayannopoulou *et al*. (2003).

Histopathological evaluation

The tumour wise distribution of myofibroma, canine transmissible venereal tumour, squamous cell carcinoma, leiomyoma and fibroma according to histopathology was 50 per cent (6/12), 25 per cent (3/12), 8.33 per cent (1/12), 8.33 per cent (1/12), 8.33 per cent (1/12), respectively. The whorl pattern with spindle cells was observed in myofibroma, leiomyoma and fibroma as reported by Patsikas *et al.* (2014) and Umamageswari *et al.* (2016). The histopathological appearance of CTVT was confirmed by spherical, polyhedral or ovoid shaped cells with enormous central nucleoli and large spherical nucleus. This was in accordance with Priyadarshini *et al.* (2021). The appearance of the squamous cell carcinoma was similar to the description suggested by Nemec *et al.* (2012). The tumour was characterised by the presence of polygonal eosinophilic cells after the occasional formation of keratin pearl.

Radiography

Radiography did not show any evidence of radio-opaque lesions in lung field consistent with tumour metastasis. The abdomen was devoid of any focal radio-opaque lesion. The canines with slight to moderate constriction of rectum accounted (33.30%) (Fig. 1). The absence of the metastatic lesions in abdomen and thorax was probably due to benign nature of the tumours. This was in agreement with Katamoto *et al*. (2003), Ojeda *et al*. (2018) and Theodorou *et al.* (2010). Despite the metastatic nature of squamous cell carcinoma, no pulmonary metastasis was

Table 1. Haematological parameters before treatment

Blood parameters	Range	Mean ± SE
TLC (x 10^6/µL)	9.2 - 27.5	15.25 ± 1.89
TEC (x 10^6/µL)	2.4 - 6.9	4.90 ± 0.37
Lymphocyte (%)	13.4 – 37.7	28.05 ± 4.30
Granulocyte (%)	21.7 – 80.5	63.33 ± 4.30
Monocyte (%)	6.1 – 12.4	8.75 ± 0.58
Hb (g/dL)	4 – 13.4	10.30 ± 0.88
VPRC (%)	16 – 38.3	31.35 ± 1.90
BUN (mg/dL)	7.4 - 17.38	14.37 ± 1.25
Creatinine (mg/dL)	0.6 - 1.68	1.17 ± 0.10
ALP (IU/L)	141 - 386	229.70 ± 22.73

Table 2. Correlation between quantity of positive cells and scores of immunohistochemical staining of ERα and Ki-67

Variable	Correlation coefficient
ERα	1 *
Ki-67	1*

(*Significant at 1 % level)

Table 3. Combined score of immunohistochemical staining

Sl No.	Erα	Ki-67
Myofibroma	5	3
Leiomyoma	5	4
Fibroma	7	2
CTVT	0	7
SCC	0	8

observed, which was in contrast to Wong *et al*. (2002), who reported pulmonary and lymphatic metastasis of the same tumour in murine studies. There were no secondary neoplasms in any animal, which could be due to the solitary nature of majority of the tumours. A similar observation was reported by Kydd and Burnie (1986).

Immunohistochemical evaluation

The correlations between expressions of ERα and Ki67 were assessed to derive the status of malignancy in these tumour and nature of dominance of oestrogen receptor in genital neoplasms. The correlation between immunoscores of ERα for intensity and the quantity of the positive cells were highly significant ($p < 0.01$) suggesting that intensity and the quantity of the positive cells enhanced simultaneously(Table 2, Fig. 2). The higher expression of ERα in majority of the tumours were suggestive of benign nature. The four neoplasms with no expression may have been due to their origin *viz*., neoplasms with histiocytic (n = 3) and epithelial origin (n=1) were presented without positive cells. These findings were in agreement with Ganguly *et al*. (2016), who suggested the absence of ERα in these neoplasms.

Table 2. Correlation between scores of immunohistochemical staining of ERα and Ki-67

Variable	Ki-67
Erα	-0.712*

(* - Significant at 5 % level)

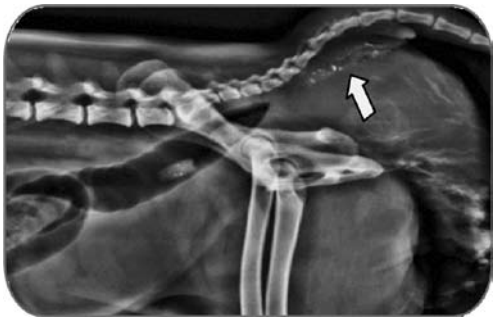

Fig. 1. Radiography of perineal region showing rectal constriction

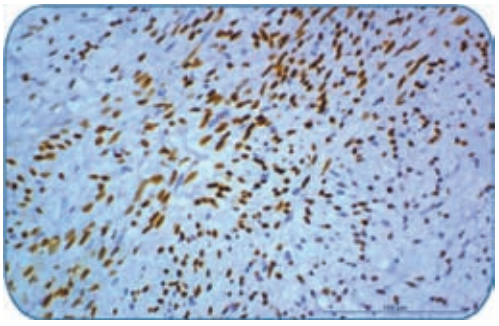

Fig. 2. ERα expression in a myofibroma

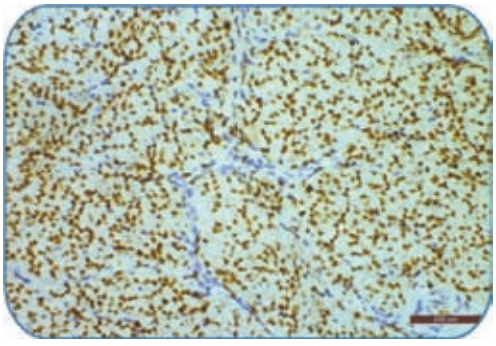

Fig. 3. Positive Ki-67 expression in Squamous cell carcinoma

Correlation of quality of intensity of positively stained cells and quantity of positively stained cells score of Ki-67 in the tumour tissues was significant ($p < 0.01$) as represented, suggesting a trend of linear

inclination in intensity and the quantity of the positive cells. The expression of Ki-67 was highest in SCC followed by CTVT, suggestive of malignant nature of the neoplasms (Fig. 3). Correlation between the intensity of positively stained cells of ERα and quantity of positively stained cells of Ki-67 in the tumour tissues was statistically significant ($p < 0.05$) but correlated negatively as represented (Table 3). These observations suggest that ERα expression is higher in benign neoplasms and reduces during malignant transformation, contrary to this, Ki-67 expression behaves in an invert manner by being highly expressed in mitotically active neoplasms and sparingly expressed in benign neoplasms with some variations in highly oestrogen dependent tumours (Table 4).

Conclusion

Thus, it could be concluded that loss of ERα expression in canine genital neoplasm contributes to its transformation from benign to its malignant counterpart. The later could be estimated by deriving the expression of Ki-67 receptors in the neoplasms. Higher Ki-67 expression was a feature of malignant neoplasms of the genital tract like the squamous cell carcinoma. Ki-67, a versatile marker for proliferation could be assessed to know the degree of proliferation possessed by the neoplasm. A correlation between these two molecular markers may provide better clarity in neoplasms with borderline malignant characteristics.

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Conflict of interest

The authors declare that they have no conflict of interest.

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