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ORIGINAL ARTICLE

The number of ERα and PR in the mammary glands of bitches with and without tumor mass using immunohistochemical assay

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Abstract The objectives of this study were to evaluate the expressions of estrogen receptors alpha (ERα) and progesterone receptors (PR) in histologically normal and tumoral canine mammary tissues within the same bitch and to correlate between the immunohistochemical staining scores of both receptors and duration of tumor growth. Twelve tumoral and contralateral normal mammary tissues, both determined by histology were surgically obtained from 12 dogs. The expressions of ERα and PR were evaluated by avidin–biotin–peroxidase complex (ABC) method and ERα and PR scores were calculated. The expressions of the ERα and the PR between normal and tumoral mammary tissues were not significantly different. The PR expression and duration of tumor growth showed a significantly inverse correlation. A positive correlation was found between the number of the ERα and the PR in both normal and tumoral mammary tissues which may indicate that either ERα or PR could be used as a prediction of hormonal therapy parameter in dogs. However, the information of ERα and PR status as well as hormonal therapy is not routinely available in veterinary medicine and it appears that half of the mammary tumors from the present study are hormone-independent tumors (ERα and PR negative). Therefore, the treatment of canine mammary tumor by hormonal therapy should be carefully considered.

Keywords Bitches · Mammary gland · ERα · PR · Immunohistochemistry

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Introduction

Mammary tumors are the second most common neoplasms in dogs, next to skin tumors, and approximately 40–50% of these tumors are malignant (Fanton and Withrow 1981). It can be a cause of death if the tumor cells are spread to other organs. The evidence of mammary tumors is thought to be dependent on the endocrine status of the host (Yamagami et al. 1996). Estrogen and progesterone may play an important role in the pathogenesis of canine mammary tumors (Fanton and Withrow 1981; MacEwen 1990; Martin de las Mulas et al. 2004). The ovarian steroid hormones, estrogen and progesterone, exert their actions on target cells predominantly through the binding and activation of the estrogen receptor (ER) and progesterone receptor (PR) (Brosens et al. 2004). There are two estrogen receptors, estrogen receptor alpha and beta (ERα and ERβ respectively) (Martin de las Mulas et al. 2004). In female, ERα is abundant in classical estrogen target tissue including mammary gland, while ER-β is highly expressed in non-classical estrogen target tissue such as ovarian follicle (Weihua et al. 2003).

Analysis of the expressions of ERα and PR has become accepted as useful tools in prognosis and prediction of hormonal therapy response in human breast cancer (Snead et al. 1993). ERα and PR positive breast tumor have been shown to depend on estrogen for growth and should respond to hormonal therapy (Jensen and Desombre

1997). On the other hand, ERα and PR negative tumors would be less dependent on estrogen and therefore less responsive to hormonal therapy (Osborne et al. 2005). In dogs, steroid hormone receptors also have been detected in normal mammary tissue and mammary tumors (Donnay et al. 1995a; Geraldes et al. 2000; Graham et al. 1999; Hamilton et al. 1977; MacEwen et al. 1982; Nieto et al. 2000). From these earlier studies, different results of ERα and PR status in normal canine mammary tissues as well as mammary tumors were reported. Regarding immunohistochemistry, the available data on steroid receptors of normal mammary and tumoral tissue was done by collecting samples from different bitches. Endocrine status may vary among individual bitch which will affect the expression of steroid hormone receptors in mammary tissues (Donnay et al. 1995b).

Moreover, there are no previous reports, from data using the immunohistochemical technique on the detection of ERα and PR in normal and tumoral mammary tissues within the same bitch. Therefore, the objectives of this study were to evaluate the differences between immunohistologically ERα and PR expression in normal mammary tissue and mammary tumor from the same bitch and to determine the relation between ERα and PR scores and the duration of tumor growth.

Materials and methods

Animals

A total of 12 adult intact bitches of various breeds bearing mammary tumors were included in this study. All bitches were referred to the obstetrics and gynecology unit, Chulalongkorn University small animal teaching hospital for treatment of mammary tumor by surgical excision. In this present study, only adenoma and adenocarcinoma types were selected to represent for benign and malignant tumors respectively. Moreover, in order to avoid the different endocrine status among individual bitch, mammary tumor and normal mammary tissues were collected from the same bitch.

Collection of samples

Both mammary tumors and contralateral normal mammary tissues from the same bitch were obtained during the surgical procedure. Immediately after surgical excision, they were fixed in 4% paraformaldehyde up to 48 h. Thereafter they were dehydrated, embedded in paraffin and 4-μm thick sections were cut from and placed on Polysine™ slides (Menzel-Glazer, Germany). One section was stained with hematoxylin and eosin, and the other sections were used for immunohistochemistry.

Histological examination

Sections of each tumor, stained with hematoxylin and eosin, were evaluated microscopically and classified using the diagnostic criteria proposed by the World Health Organization classification of tumor in domestic animals (Hampe and Misdorp 1974).

Immunohistochemistry of estrogen and progesterone receptors

Before immunohistochemistry, the specimens were deparaffinized in xylene and rehydrated in graded alcohol. The immunohistochemical protocol was modified from Sukjumlong et al. (2003). Antigen retrieval technique was effected by means of heating in the microwave with 0.01 M citrate buffer, pH 6.0 in a microwave at high power (750 W) for 15 min (5 min×3). A standard avidin–biotin immunoperoxidase technique (Vectastain® ABC kit, Vector Laboratories, USA) was applied to detect ERα and PR proteins. The primary antibodies used were mouse monoclonal antibody to ERα (Novocastra NCL-ER-LH2, clone CC4-5, dilution 1:50) and PR (Immunotech, clone 10A9, dilution 1:100) for 2 h in a humidity chamber at room temperature.

The samples that were treated with non-immune serum, instead of the specific antibody, were run as negative controls. Normal canine uterus taken at estrus and known to express ERα and PR was served as positive controls.

In the final step, the color was developed with a freshly prepared solution of 3, 3′-diaminobenzidine (DAB kit, Vector Laboratories, USA). All sections were counterstained with Mayer's hematoxylin, dehydrated and mounted with glycerine gelatin for investigation under a light microscope.

Evaluation of immunohistochemical data

The immunostaining for ERα and PR was assessed on the basis of visually estimated percentage of neoplastic cells and alveolar and tubular epithelial cells of normal mammary tissues with positive nuclear staining (brown nuclei) by counting 1,000 cells in 10 to 20 fields per histological sections with nuclear staining for ERα and PR. The human score system was used. ERα score and PR score were calculated as $P_1+(2\times P_2)+(3\times P_3)$ where P_1, P_2, and P_3 are the estimated percentages of positive nuclei with low (P_1), medium (P_2), and high (P_3) intensity of immunostaining color (Snead et al. 1993).

Statistical analysis

The differences between the expressions of histologically ERα and PR on normal mammary tissues and mammary

tumors were evaluated using paired *t*-test and correlation between ERα and PR scores and duration of tumor growth within the groups of bitch were evaluated using Spearman's rank correlation coefficient. A probability of error (*P* value)≤0.05 was selected as significant.

Results

Of the 12 tumors, four were histologically malignant (adenocarcinoma) and eight were benign (adenoma). All of tumor masses located on the caudal mammary glands of both sites (left and right third, fourth, and fifth). The age number and site of mammary samples of the bitches are shown in Table 1 with ERα score and PR score in Table 2. In general, ERα and PR immunostaining were localized in the nuclei of normal, benign, and malignant epithelial cells (Figs. 1 and 2). In the negative controls, all nuclei were negatively stained.

The ERα and PR immunostaining scores (mean±SD) in normal and tumoral mammary tissues were shown in Table 3. The immunostaining scores of ERα and PR in normal mammary tissues were higher than those in mammary tumors, though they were not significantly different.

The ERα and PR scores were positively correlated (Spearman's coefficient ($P \leq 0.01$) in both normal and tumoral mammary tissues. The expressions of ERα and PR were not significantly correlated to the duration of tumor growth and tumor size. However, regarding the groups of benign and malignant tumors separately, PR score significantly decreased when increased duration of tumor growth ($P \leq 0.05$) in malignant tumors, but not with ERα scores. In malignant tumors, ERα and PR score decreased when tumor size increased, though not significant.

Table 2 Estrogen receptor (ERα) and progesterone receptor (PR) scores in histologically normal and tumoral mammary tissues in the same bitch

No.	Normal mammary tissue		Mammary tumor tissue	
	ERα score	PR score	ERα score	PR score
1	43.9	43.4	0.0	0.0
2	0.0	0.0	0.0	0.0
3	30.7	137.2	28.9	16.2
4	0.0	0.0	0.0	0.0
5	17.1	18.6	13.7	57.0
6	0.0	46.3	0.0	38.5
7	0.0	0.0	6.4	13.3
8	0.0	0.0	0.0	0.0
9	53.0	12.1	110.4	117.2
10	49.7	40.2	0.0	0.0
11	0.0	0.0	0.0	0.0
12	0.0	153.5	0.0	98.6

Discussion

All of the mammary tumors in this investigation were found on the posterior mammary glands. This agrees with Mann (1984) and Martin de las Mulas et al. (2004) who showed that canine mammary tumors increased in frequency from anterior and posterior gland.

The presence of ERα and PR in normal and tumoral canine mammary tissues was detected by using specific monoclonal antibodies directed against human protein. Immunoreactivity was recognized in normal tissues as well as benign and malignant tumors in which both ERα and PR were localized mainly in the nuclear area of alveolar and tubular epithelial cells. Similar immunostaining results have been found in previous studies (Geraldes et al. 2000; Nieto

Table 1 General information of 12 bitches in the study

No.	Age (years)	Growth duration (month)	Site	Diameter (cm)	Histological type
1	8	6	R 3, 4	15	Adenoma
2	6	1	R 5	2	Adenoma
3	14	2	R 4, 5	10	Adenoma
4	8	4	R 5	13	Adenoma
5	3	3	L 5	7.5	Adenoma
6	2	0.5	L 4	6	Adenoma
7	6	2	L 3–5	1.5	Adenoma
8	5	12	R 3–5	6.25	Adenoma
9	10	12	R 5	10	Adenocarcinoma
10	10	12	L 4	20	Adenocarcinoma
11	8	12	L 4, 5	7.5	Adenocarcinoma
12	10	12	R 3	1.5	Adenocarcinoma

R Right side, *L* left side

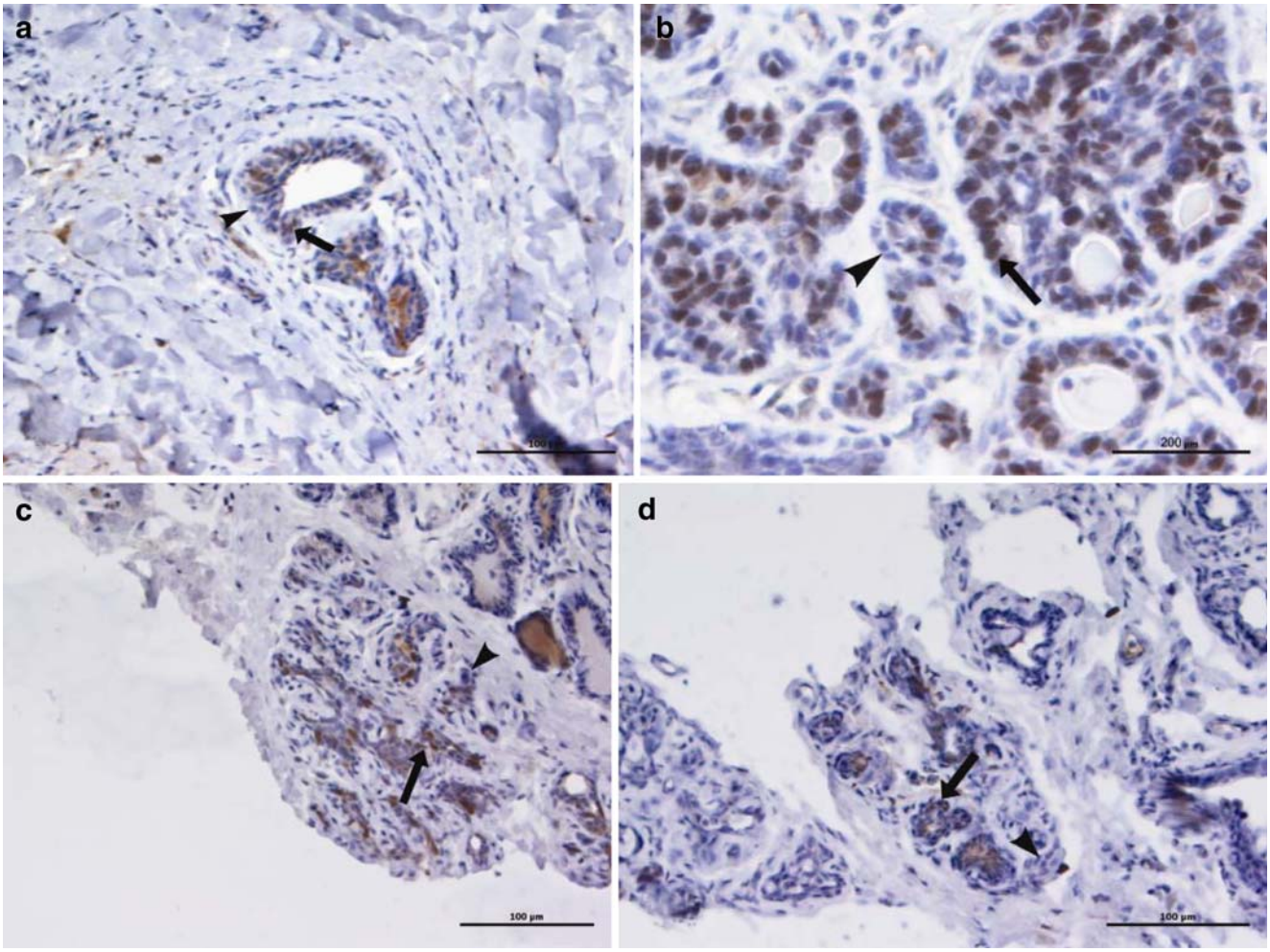

Fig. 1 Immunostaining in canine mammary tissue within the same bitch, **a** and **b** represent ERα immunostaining in normal mammary tissue and benign tumoral tissue respectively. **c** and **d** represent PR immunostaining in normal mammary tissue and benign tumoral tissue respectively. *Arrow* head and *arrow* show, respectively, negative and positive immunostaining cells

et al. 2000). Moreover, by using biochemical assay, earlier studies have reported the similar results that ERα and PR could be detected in both normal and tumoral canine mammary tissues (Donnay et al. 1995a; Elling and Ungemach 1983; Hamilton et al. 1977; MacEwen et al. 1982; Rutteman et al. 1988; Sartin et al. 1992).

The expressions of the ERα and the PR in normal and tumoral mammary tissues in this study were not significantly different, which suggested similar regulation of both steroid receptor expressions in those tissues within the same bitch. Millanta et al. (2005) reported similar results that the immunohistochemical detection of ERα and PR in canine normal mammary tissue and benign mammary tumor from different dogs are not significantly different, but they are significantly higher than in carcinoma. Conversely, Donnay et al. (1995a) found that mean ER concentrations were significantly higher in normal than tumoral tissue, but PR concentrations were significantly lower in normal than in tumor. Tumor heterogeneity and the use of a different method for detection of these steroid hormone receptors may be responsible for this lack of agreement. In veterinary practice, in case of mammary tumors, the first recommendation is to remove tumor masses and the affected glands (Mann 1984). As ovarian steroid hormone may have the influence on steroid receptor status which could affect mammary gland tumors, therefore, ovariohysterectomy concurrently performed with tumor removal may be of useful to eradicate the effect of ovarian steroid hormone on the expression of the receptor.

From the present study, it seems that normal mammary glands have higher mean percentage of ERα as well as PR than mammary tumors from the same bitch, though this was not significant. Our results agree with an earlier study which reported a decrease in ERα levels from healthy to neoplastic mammary tissues (Millanta et al. 2005). However, the lack of statistical significance from the present

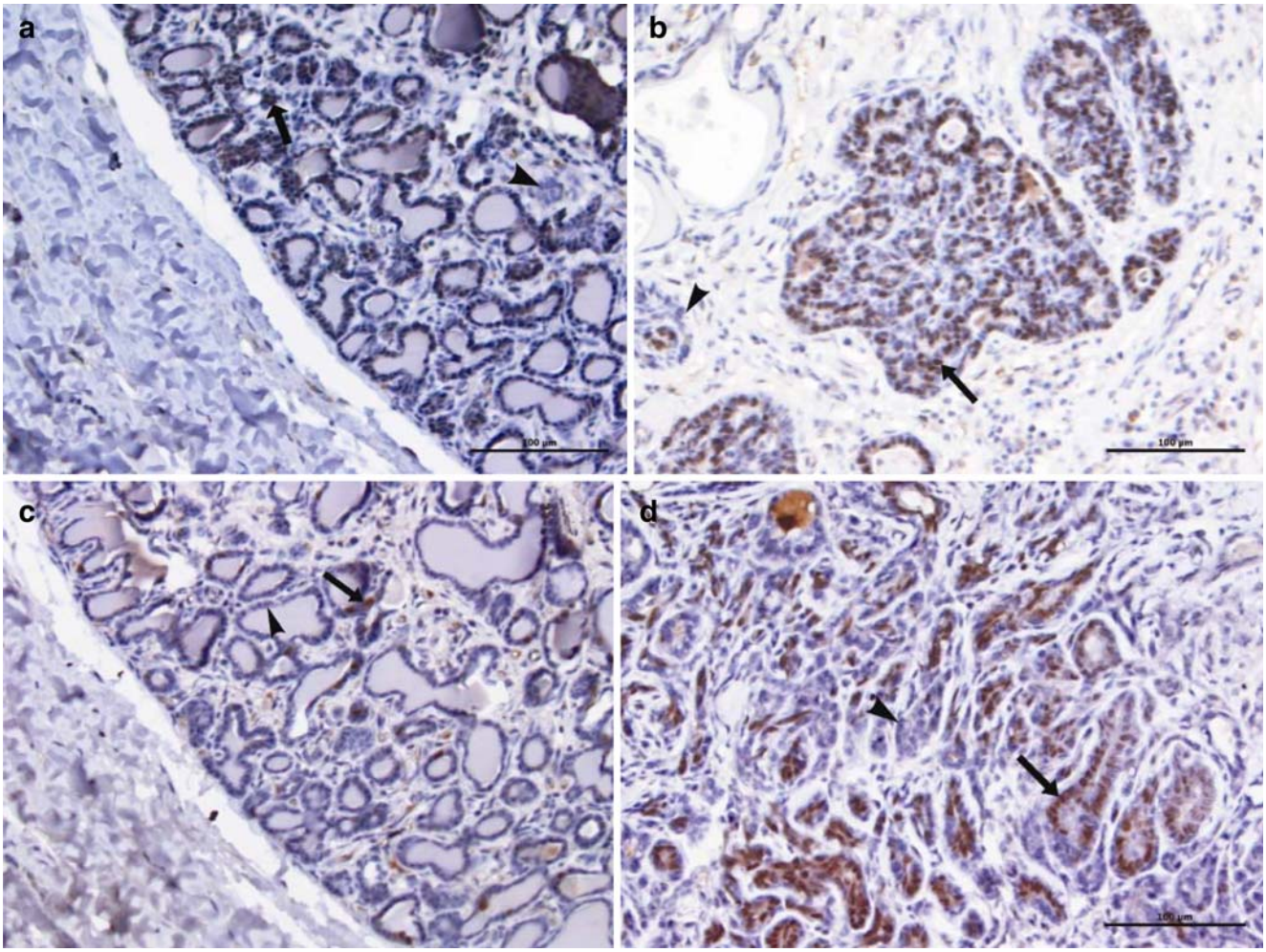

Fig. 2 Immunostaining in canine mammary tissue within the same bitch, **a** and **b** represent ERα immunostaining in normal mammary tissue and malignant tumoral tissue respectively. **c** and **d** represent PR immunostaining in normal mammary tissue and malignant tumoral tissue respectively. *Arrow* head and *arrow* show, respectively, negative and positive immunostaining cells

study could be related to the high number of ERα and PR negative results in the normal mammary tissue studied. In contrast, Geraldes et al. (2000) reported that all benign tumors in dogs analyzed contained PR while high percentage of normal as well as mammary tumors from the present study was PR negative. The difference between these may be attributed to different hormonal status of the bitches investigated as suggested by Donnay et al. (1995b) which demonstrated the significantly high concentration of ER during luteal phase and high concentration of PR during anestrus in normal mammary tissue, however, it was not significant in tumor tissues. Moreover, it appears that half of the mammary tumors from the present study are hormone-independent tumors (ERα and PR negative). Therefore, the treatment of canine mammary tumor by hormonal therapy should be carefully considered. Besides ovarian steroid hormones, estrogen and progesterone, it has been reported that other growth factors such as epidermal growth factor, insulin-liked growth factor-I (IGF-I), and transforming growth factor (Donnay et al. 1995a; Martin de las Mulas et al. 2004; Mol et al. 1997) may influence the pathogenesis of mammary gland tumors by induced epithelial cell proliferation and differentiation as well as high-fat diet (Nieto et al. 2000; Sonnenschein et al. 1991) may influence by increased level of IGF-I.

Table 3 Summary of ERα and PR scores in normal mammary tissues and mammary tumors from 12 bitches (mean±SD)

Mammary tissues	ERα score	PR score
Normal mammary (n=12)	16.20±21.93[a]	37.61±53.55[a]
Mammary tumors (n=12)	13.28±31.82[a]	28.40±41.51[a]
Overall significance	P<0.05	P<0.05

Mean (±SD) within the same column followed by the different superscript letters are significantly different (P≤0.05)

A positive correlation was found between the number of the ERα and the PR in both normal and tumoral mammary tissues. This was in agreement with Donnay et al. (1995a) and could be related to induction of PR by estrogen binding to the ER (Elling and Ungemach 1983; Horwitz and McGuire 1978a). In human breast cancer, the analysis of the expressions of ERα and PR has become accepted as useful tools in prognosis and prediction of hormonal therapy (Iwase et al. 2003; Pichon et al. 1980). It was shown that a high content of ERα in a tumor is associated with a better prognosis and allows hormonal treatment after surgical excision.

In dogs, the presence of ER and PR also seem to relate to a better prognosis (Sartin et al. 1992), therefore, the positive correlation between ERα and PR from the present study may indicate that either ERα or PR could be used as a prediction of hormonal therapy parameter in dogs. Jensen and Desombre (1997) state that all patients with human breast cancer and that have ERα and/or PR positive should received hormonal therapy. Moreover, PR was defined as an ER-regulated gene product; therefore the presence of PR may indicate that the ER is functioning (Horwitz and McGuire 1978b). Therefore, both ERα and PR positive tumors would respond to hormonal therapy more than the ERα positive tumor without PR (Osborne et al. 2005). However, the information about ERα and PR status as well as hormonal therapy is not routinely available in veterinary practice.

From the present study, PR score and duration of tumor growth showed a significant inverse correlation. This finding reinforces the hypothesis that the development of breast cancer is paralleled by a progressive decrease in hormone dependence. The progression towards malignancy in spontaneous canine mammary tumor is accompanied by a decrease in hormone steroid dependency and an increase in autonomous growth (Rutteman et al. 1988; Schmitt 1995).

Four bitches from the study did not demonstrated either ERα or PR expression at all, this may due to several factors such as the lack of steroid receptors, the undetected receptors by monoclonal antibody but might still be function or non-functional statuses of the receptors.

In conclusion, the present study showed that both normal and tumoral mammary tissues from bitches have heterogeneous expression of steroid receptors, ERα and PR. In the same bitch, normal and tumoral mammary tissues demonstrated the same pattern of steroid receptor expression. The results from this study may be used for the further investigation of the recurrence of tumors in the non-removal mammary gland and also useful in aiding the selection of the appropriate therapy for canine mammary tumors after surgical treatment. However, hormonal therapy should be applied with caution in canine mammary tumors as approximately half of the mammary tumors investigated from the present study can be categorized as hormone-independent tumors (ERα negative/PR negative). This may be the genuine steroid-negative receptors or non-functional steroid receptors which should be consequently investigated. Further studies on hormonal therapy for mammary gland tumors would be useful in veterinary medicine.

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