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Immunohistochemical Evaluation of Ovarian Hormonal Receptors in Canine Mammary Tumors

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ABSTRACT

Background: In female dogs, ovarian hormones have a crucial role in the pathogenesis of mammary tumors. Ovarian hormones interact with nuclear receptors, estrogen receptor α (ERα) and β (ERβ), progesteron receptor (PR) and androgen receptor (AR) respectively. The aim of this study was to verify the existence of correlations between biological behaviour and immunohistochemical expression of estrogen receptor α (ERα) and β (ERβ), progesterone receptor (PR) and androgen receptor (AR) in canine mammary tumors. A total of sixty-four tumors were examined. **Results:** The expression of every receptor was higher in normal tissue and benign tumors than in malignant neoplasm. Among malignancies, the lowest levels of every receptor were detected in high grade carcinomas ($p < 0.01$). Lower levels of ERα and PR were associated with regional ($p < 0.01$) and/or distant ($p < 0.05$) metastasis. A lower expression of ERβ was found in carcinomas with nodal positive status ($p < 0.05$). High level of AR seemed weakly associated with the development of distant metastasis ($p > 0.05$). **Conclusions:** The expression of ERα and/or PR showed the positive prognostic value for the Spearman's rank test ($p < 0.01$). ERβ also displayed a positive prognostic significance ($p < 0.05$). The levels of AR were inversely correlated only with grading of a slight positive correlation with metastatic power of carcinoma ($p > 0.05$). In human breast carcinoma, AR seems to be involved in metastatic development by up-regulation of metalloprotease of matrix (MMP). Therefore, evaluating the correlation among the presence of AR, expression of MMP and appearance of distant metastasis also in canine mammary tumors could be very interesting.

Keywords: Ovarian Hormonal Receptors; Mammary Tumours; Dog; Immunohistochemistry

1. Introduction

In female dogs, it is known that the crucial role of ovarian hormones in the pathogenesis of mammary tumors [1-5].

Ovarian hormones, estrogen, progesteron and androgen, play their functions by nuclear receptors as estrogen receptor α (ERα) and β (ERβ), progesteron receptor (PR) and androgen receptor (AR) respectively. The immunohistochemical evaluation of hormonal receptor status is a very useful tool in human breast cancer management [6-8].

In veterinary medicine, there is little knowledge of the role of ERα, ERβ, PR and AR in canine mammary tumors. It is well-known that the presence of ERα in canine mammary tumors means a good prognostic factor; in fact, the expression of ERα appears higher in normal and hyperplastic tissue as well as in benign tumors than in carcinomas [4,9-11]. Therefore, the presence of important cellular atypia, high mitotic index and regional/distant metastasis, is related with a lower expression of Erα [9,10,12,13]. Some epidemiological and clinical variables like age, spaying and parity status, pseudopregnancy and hormonal treatment to control oestrus were associated with the content of ERα in canine normal and neoplastic mammary tissue [9,10,14-16].

ERβ is less studied than ERα. We know that ERβ is expressed by the majority of normal epithelial cells in canine mammary gland, but we can find neoplastic mammary cells ERβ+. There are contradictory results concerning the significance of ERβ. Martin de la Mulas *et al*. (2004) [17] showed that the ERβ+ mammary tumors are usually benign or with a low grade of malignancy. On the other hand, the inflammatory mammary carcinomas, a group of very aggressive tumors, are often ERβ+ and ERα− [18]. There are no studies concerning the relationship between expression of ERβ and epidemiological variables.

The presence of PR is considered as a very good

prognostic factor in canine mammary tumors. In fact PR+ tumors are often benign and a little percentage is malignant, with low mitotic index and without metastatic invasion [4,9,12,13,17,19].

The role of AR is still unknown. Illera *et al.* (2006) [18] demonstrated the presence of this receptor in normal and neoplastic canine mammary tissue. A high expression of AR appeared especially in infiltrating carcinoma with metastasis and in inflammatory carcinoma. Therefore AR might be involved in the tumoral invasion process.

The aim of this study was to evaluate the correlation between biological behaviour and immunohistochemical expression of estrogen *α* and *β*, progesteron and androgen receptors in canine mammary tumors with 12 months follow-up.

2. Methods

2.1. Tissues

Sixty bitches (aged between 4 - 12 years) with mammary neoformation were included in this study. Information concerning age, breed, history of ovariectomy, hormonal prevention of oestrus, number of full-term pregnancies and presence of psudopregnancy were obtained from theirs owners. The bitches were clinically examined and thoracic X-ray was performed to reveal metastasis. Clinical staging was determined according to TNM classification of World Health Organization [20]. Only dogs without distant metastasis (M0) were included in the study. Partial or total mastectomy was carried out and the whole tissue, fixed in 10% buffered neutral formalin, was sent to Laboratory of Animal Pathology, University of Camerino (Italy). The animals were followed up during a year after the surgery. The plan of follow-up included complete clinical examination and thoracic X-ray. Presence of relapse and/or pulmonary metastases was recorded and the cause of death was confirmed by post-mortem examination.

For this experimental work were not sacrificed animals but were used biopsies taken as part of a veterinary visit.

2.2. Immunohistochemistry

A representative portion of each sample, fixed in formalin and embedded in paraffin wax, was cut in 4 μm-thick sections; one slide was stained with haematoxylin-eosin, the others were used for the immunohistochemical analysis. The samples were histologically classified according to criteria of WHO [21]. The grading was performed through the method of Kurzman and Gilbertson (1986) [22]. The regional lymph nodes were analyzed too. The immunohistochemistry was carried out by the Streptavidin-Biotin-Peroxidase (ABC) method. After microwave

antigen unmasking (8' at 650 W for two times) and inhibition of endogenous peroxidase activity (60' with H_2O_2 in 0%, 3% distilled water), the slides were incubated overnight with polyclonal antibodies against ER*α* (1:100, Santa Cruz Biotech), ER*β* (1:100, Santa Cruz Biotech), PR (1:150, Santa Cruz Biotech) and AR (1:100, Santa Cruz Biotech). Positive controls have consisted in normal canine mammary tissue for ER*α*, ER*β* and PR and normal canine prostate for AR. Negative controls were made by replacing the primary antibodies with three saline buffer TBS. The expression of every antibody in each sample was quantified by counting the number of positive nuclei in 100 cells of 10 high-power representative fields. Data were expressed as mean percentage ± SD and the negative cut-off point was assessed to 5%.

2.3. Statistical Analysis

Statistical analysis was performed using Spearman's rank test and the Student's t-test. $p < 0.05$ was regarded as significant.

3. Results and Discussion

A total of sixty bitches were involved in this study. Forty-two bitches (70%) were intact whereas four (6%) were spayed before the second oestrus. Sixteen dogs (26%) developed recurrences (12 up to 6 months and 4 between 6 and 12 months); six dogs (10%) developed distant metastases (two up to 6 months and four between 6 and 12 months) and all these dog died during the follow-up period.

We have examined 64 mammary samples: 8 benign lesions (4 epithelial hyperplasia and 4 adenomas) and 56 carcinomas (two "*in situ*" carcinomas and 54 invasive carcinomas). Histological classification and grading are summarized in **Table 1**. Regional lymph nodes were positive for metastases in 16 samples (27%).

Table 1. Histological classification and grading of mammary neoformation.

		S.S.	S.T.P.	Complex	Total
Total Malignant		**14**	**14**	**28**	**56**
Without LN Met		8	8	24	40
	Grade I	0	8	10	
	Grade II	4	0	14	
	Grade III	4	0	0	
With LN Met		6	6	4	16
	Grade I	0	0	0	
	Grade II	2	4	0	
	Grade III	4	2	4	
Total Benign		**0**	**8**	**0**	**8**
Total Number		**14**	**22**	**28**	**64**

LN Met: lymph nodal metastases; S.S.: simple solid type; S.T.P.: simple tubulopapillary type.

Immunohistochemically, we have found specific reaction to ERα, ERβ, PR and AR in the nuclei of normal and neoplastic epithelial and myoepithelial mammary cells. The cartilaginous cells in the complex/mixed tumors were always negative. In some samples, a minimal citoplasmatic staining can be found.

Normal lobules and hyperplastic areas exhibited a strong, homogeneous positiveness towards to the four receptors. Normal myoepithelial cells were always positive to every receptor (**Figure 1**).

In neoplastic tissue the intensity of reaction appeared very heterogeneous among the several samples and in the same case too. In fact, many tumors showed areas strongly positive close to others hardly negative. Immunostaining to ERα, ERβ, PR and AR was uniform and intense in benign tumors. All the cancer except one (96%) were positive to ERα but a fall of positiveness with the rise of malignancy ($p < 0.01$) was shown. In four cases neoplastic cells in regional nodes were ERα+. ERβ was detected in 44 carcinomas (74%). An inverse correlation between percentage of positive cells and tumoral grading ($p < 0.01$) were found. Moreover four of the 12 negative cases were classified as moderately differentiated carcinomas and the others as high-grade carcinomas. Just two metas-

tatic lymph nodes exhibited immunoreaction to ERβ. The ratio ERα:ERβ did not give significant correlation with grading or prognostic factors. PR was revealed in all the carcinomas except two (96%). The mean values of positiveness are reported in **Table 2** and in **Figures 2** and **3**.

There was a significant correlation between lower number of positive cells and higher tumoral grade ($p < 0.01$). PR was detected in two of 16 metastatic lymph nodes (12%). Specific reaction to AR was found in every cancer but low-grade carcinomas showed a higher positiveness than moderately or poorly differentiated carcinomas ($p < 0.01$). Metastatic cells in lymph nodes were AR+ in four cases (25%). Two lymph nodes were positives to all the receptors whereas just one was ERα/AR+. We also assessed the number of positive neoplastic myoepithelial cells in complex tumors. The expression of ERα was almost uniform in the three classes of malignancy and not correlated with the grading ($p > 0.05$). ERβ appeared less detected in high-grade than low-grade carcinomas but this correlation did not come to the statistical significance ($p > 0.05$). A weak, non statistical significant, direct relationship was observed between grading and myoepithelial expression of PR ($p > 0.05$). On the other hand, we found a strong inverse correlation
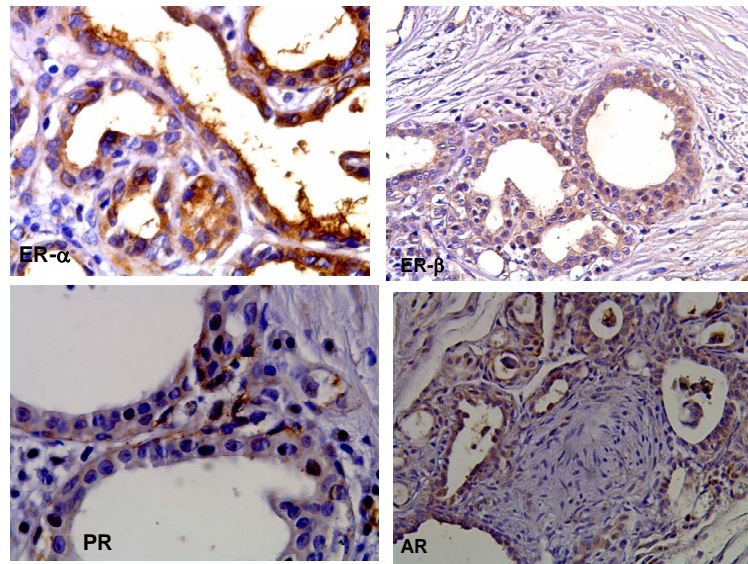

Figure 1. Normal mammary tissue. Immunostain to ER-*α*, ER-*β*, PR, AR. ABC, 40×. Immunopositivity for all markers.

Table 2. ERα, ERα, PR and AR index (mean ± SD) in canine mammary tumors.

Numer of samples		Indices (means ± SD)			
		ERα	ERβ	PR	AR
Benign	16	92.1 ± 5.4	68.3 ± 12.6	83.9 ± 12.1	72.8 ± 9.1
Malignant	56	55.4 ± 29.4	38.7 ± 28.3	46.1 ± 30.8	43.5 ± 23.2
HG I	18	80.7 ± 15.7	66 ± 18.2	79.1 ± 12.7	65.4 ± 19.7
HG II	22	59.7 ± 10.7	34.7 ± 13.4	41.5 ± 13.9	35.3 ± 16.9
HG III	16	25.8 ± 14.5	13.6 ± 13.5	15.3 ± 6.7	30.1 ± 17.4

HG: histological grade.
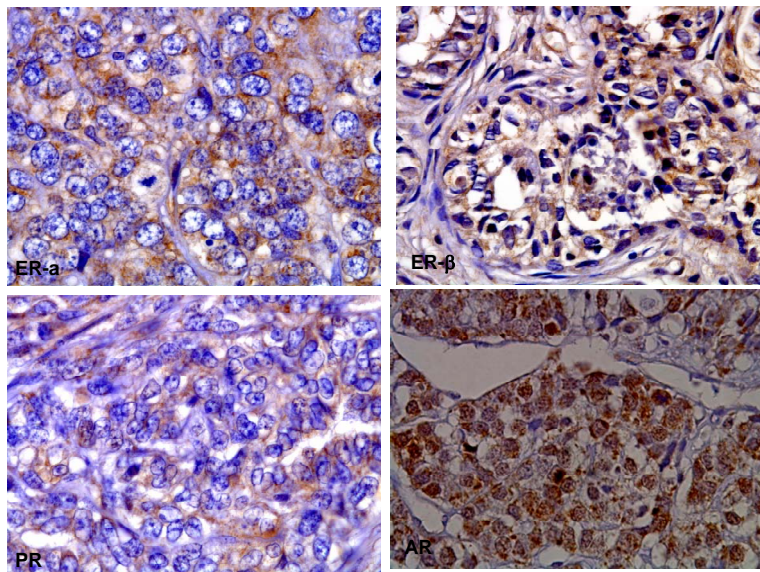
Figure 2. Simple carcinoma. Immunostain to ER-*α*, ER-*β*, PR, AR. ABC, 40×. Inverse correlation between percentage of positive cells and tumoral grading.
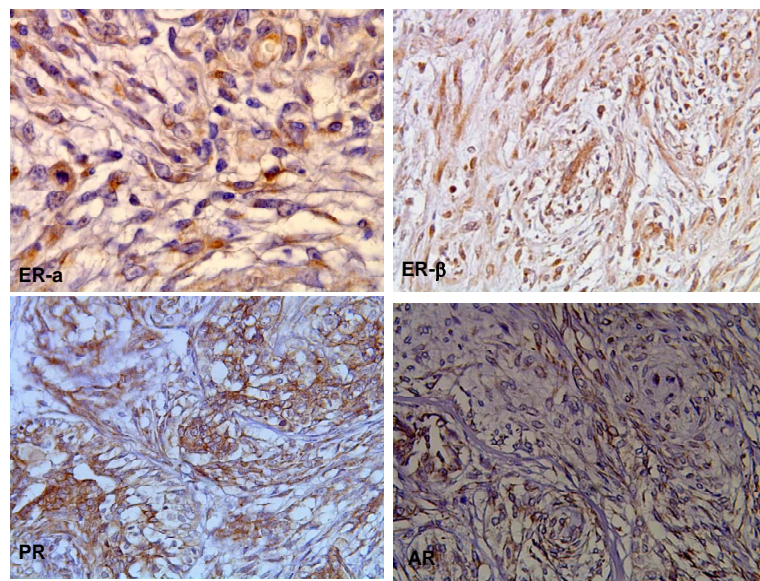
Figure 3. Complex carcinoma. Immunostain to ER-*α*, ER-*β*, PR, AR. ABC, 40×. Inverse correlation between percentage of positive cells and tumoral grading.

between AR expression in myoepithelial cells and tumoral grading (p < 0.01). The ratio epithelial:myoepithelial positive cells did not show any significance. We did not find association between the immunohistochemical expression of hormonal receptors and epidemiological and clinical variables or between grading and spaying status. However the tumors of the bitches less than 8 years of age exhibited a higher expression of hormonal receptors, significant only for ER*α* (p < 0.05). A lack of ER*α*/ER*β*/PR expression was observed in presence of lymph node metastases (p < 0.01). On the other hand the expression of AR was nearly equal in the two groups (p >

0.05). Pulmonary metastases were associated with a significant lower ER*α* presence (p < 0.02) and a reduced but non-significant expression of ER*β* and PR (p > 0.05). The number of AR+ cells whereas appeared slightly higher in tumors with distant metastases (p > 0.05). Recurrences were associated with a lower expression of ER*α*, ER*β* and PR (non significant, p > 0.05). AR also seemed less detected in recurrent tumors but the difference between the two groups were much lower (p > 0.05). Eventually, ER*α* and PR appeared to be good indicators of Spearman's rank test (p < 0.01) and ERB too (p < 0.05). AR instead did not show significant information

concerning to Spearman's rank test (p > 0.05).

Some interesting findings have come out from this study. The spaying has a protective role against the canine mammary cancerogenesis. High level of circulating estrogens during the lifespan can improve the risk of mammary tumors [14,22]. In this study the majority (70%) of bitches were intact whereas only 4 (6%) were spayed before the second years of age. However, the grade of malignancy appeared without any correlation with spaying status or age at the time of diagnosis. Levels of ovarian hormone receptors were assessed by immunohistochemical assay. Specific nuclear and perinucleare reaction to ERα, ERβ, PR and AR was observed in normal and neoplastic epithelial and myoepithelial cells as well as in few stromal cells. Some cells also showed a weak cytoplasmatic staining; this report is attributed to a trouble of nuclear receptorial transport and was showed by other Authors [12,23]. The intensity of staining appeared very heterogeneous in the majority of tumors with areas strongly positive close to other nearly negative. This event may be due to a differentiated cellular activity [24,25].

Several association among clinical progress of tumor, grading and receptorial expression exist. Status of spaying, pregnancy or pseudopregnancy and hormonal treatments to control oestrus was not related with the levels of receptors. On the other hand, the neoplasm of the animals more than 8 years of age had a lower expression of ERα, ERβ, PR and AR, with statistical significance for ERα. There are contradictory reports concerning this issue in literature. In fact either presence [9,12,14] or absences [10,15,23] of a relationship among ERα/PR expression and age, spaying status and history of pregnancy or pseudopregnancy have been reported. To date there are not studies concerning the relationship among ERβ and AR expression and epidemiological and clinical factors. Tumor size did not prove related with hormone receptors whereas the levels of ERα, ERβ and PR were lower in carcinomas with positive lymph nodes. The expression of AR instead appeared similar in the two groups without significant difference. The levels of hormone receptors showed related with the tumoral grading. Normal mammary epithelium as well as hyperplasia and adenomas expressed high levels of ERα, ERβ, PR and AR. Among malignancies, low grade carcinomas were more positive than high grade carcinoma. We also found an inverse relationship between ERα and presence of distant metastases. Eventually both ERα and PR showed indicative of DFS. The presence and the level of expression of one or both these receptors is a probably indicator of less aggressive tumoral behaviour and may have prognostic value. However ERβ also could be able to indicate a better course of the tumor even if only the relationship with lymph nodal metastases stood out. A lower expression of

AR was related with the grading alone. On the other hand, there is a little difference of expression in the two groups "lymph nodal metastases yes/no" as well as in the two groups "distant metastases yes/no". The percentage of AR+ cells even is higher in carcinoma with distant metastases than them without these ones. The same remark is evident for the groups "relapse yes/no". We can get at two hypothesis to explain that. It could be a real lack of relationship among level of AR and metastasis and/or relapse. On the other hand, the higher expression of AR than ERα/ERβ/PR in the tumors with metastases could be a sign of the metastatic power of these carcinomas. In human medicine, Gonzales *et al.* (2008) [26] found a direct relationship between AR and regional/distant metastases in breast carcinoma. This relation seems due to an up-regulation of matrix metalloproteases (MMPs) by AR and MMPs are involved in the process of tumoral invasiveness. Therefore it could be very interesting to check if in canine mammary tumors also the presence of AR is related to a higher expression of MMPs. The level of AR was clearly lower in high grade carcinomas. This fact could clash with the above-mentioned involvement of AR in the process of tumoral invasiveness. However, all the studies refer to AR+ epithelial and not myoepithelial cells. The ratio epithelial:myoepithelial positive cells also was not related with the grading.

4. Conclusion

This study confirmed that the positive prognostic importance of ERα and PR in canine mammary tissue. However, our study showed new findings about the role and expression of ERβ concerning staging and grading. Eventually we assessed the significance of the still acknowledged AR in canine mammary tumors and carried out a characterization of the receptorial status of myoepithelial cells. Further studies are envisaged to evaluate the relationship among AR and factors related to tumoral invasiveness like MMPs.

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