Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors

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Objective—To evaluate the prognostic potential of expression of hormone receptors in malignant mammary gland tumors of dogs.

Design—Cohort study.

Animals—89 female dogs with malignant mammary gland tumors and 24 female dogs with benign mammary gland tumors.

Procedures—Female dogs with malignant (n = 89 dogs) and benign (24) mammary gland tumors were evaluated to determine the prognostic value of the expression of estrogen receptor (ERα) or the progesterone receptor (PR), as determined by use of immunohistochemical methods.

Results—In this study, 68 (76.4%) and 87 (71.9%) of the 113 dogs with mammary gland tumors had expression of ERα and PR, respectively. Expression of ERα and PR was detected proportionately more frequently in benign tumors (23/24 [95.8%] and 24/24 [100%], respectively) than in malignant tumors (45/89 [50.6%] and 64/89 [71.9%]). Percentage of tumors with positive results for ERα and PR was significantly higher in tumors < 5 cm in diameter; as clinical stage I, II, or III; and without metastasis to lymph nodes or distant metastasis. However, only PR expression in tumor cells was significantly associated with 1-year survival after surgical removal of the tumor. Moreover, dogs with malignant tumors expressing ERα and PR had a significantly higher survival rate, compared with the rate for dogs with malignant tumors expressing ERα but not PR.

Conclusions and Clinical Relevance—These findings strongly suggested that expression of PR could be used as a prognostic factor for survival, especially in female dogs with malignant mammary gland tumors with ERα expression. (J Am Vet Med Assoc 2009;235:391–396)

Many tumor markers have been investigated as prognostic indicators in humans with breast cancer. Among these markers, the ER, PR, and HER-2 have been suggested as useful biomarkers for humans with breast cancer. In dogs, both benign and malignant mammary gland tumors express ERs. However, ERα and ERβ expression were found to be higher in benign tumors than in malignant tumors. Lack of PRs was associated with a high histologic grade, tumor invasion, lymph node involvement, or metastases. Therefore, the reason for failure of ERM treatment in human breast cancer patients with ERs could be attributed to a lack of PRs. Clinical responses after ERM are significantly better in human patients whose tumors express ERs and PRs than in patients whose tumors express ERs but not PRs. Moreover, lack of expression of PRs has been significantly associated with disease progression, high tumor invasion, tumor recurrence, and death. It is of interest to investigate whether PRs are an independent prognostic factor in dogs with malignant mammary gland tumors.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>ERM</td>
<td>Estrogen receptor modulator</td>
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<td>HER-2</td>
<td>c-erbB-2 receptor</td>
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<td>OHE</td>
<td>Ovariohysterectomy</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<td>PS</td>
<td>Proportion score</td>
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<tr>
<td>TNM</td>
<td>Tumor, lymph nodes, and metastasis</td>
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Biologically, estrogens and progesterones play major roles in normal development of the mammary glands but these hormones have also been implicated in tumor development. In dogs, OHE performed early in a dog’s life significantly reduces the risk of developing mammary gland tumors. Moreover, OHE performed in dogs of an advanced age may influence the protective effect against mammary gland tumors or affect survival time after surgical removal of the tumor. In another study conducted by our laboratory group, dogs with malignant mammary gland tumors with overexpression of HER-2 generally had a higher survival rate for 2 years after surgical removal of the
tumor, compared with the survival rate for dogs with mammary gland tumors with typical expression of HER-2. Because this result differed from findings in human patients with breast cancer, it indicated that additional investigation of possible features of endocrine biomarkers in mammary gland tumors of dogs was needed. The objectives of the study reported here were to evaluate the expressions of ERα and PR in mammary gland tumors of dogs by use of immunohistochemical methods, to investigate the relationship of these hormone receptors and clinicopathologic manifestations, and to analyze the prognostic potential of hormone receptors in dogs with malignant mammary gland tumors.

Materials and Methods

Animals—Dogs with mammary gland tumors (n = 113 dogs, including 89 with malignant tumors and 24 with benign tumors) were identified for use in the study. Dogs had been examined at National Chung Hsing University Veterinary Medical Teaching Hospital between January 2000 and March 2006. Dogs were eligible for inclusion when a diagnosis of a mammary gland tumor had been confirmed by histologic examination and adjuvant chemotherapy was not administered after surgical removal of the tumor. Surgical procedures performed on dogs included lumpectomy, simple mastectomy, modified radical mastectomy (regional mastectomy), or radical mastectomy (complete unilateral or bilateral mastectomy). The extent of the surgical excision was determined by the size, location, and number of tumors and the status of regional lymph nodes.

Data collection—Historical information was obtained from the medical records and used for data analysis. Information collected included breed, age, sex, body weight, size of tumor, number of tumors, location of tumor or affected mammary glands, OHE status, reason for OHE, interval between identification of tumor and surgical removal, clinical stage as determined by use of the TNM system, surgical procedure, tumor type, histologic grade as determined on the basis of the World Health Organization–Armed Forces Institute of Pathology classification system, score for a histologic grading system (ie, tubule formation, hyperchromatism and mitoses, and irregular size and shape of nuclei) of canine mammary gland carcinomas,20,21 and overall survival time.

For all dogs, follow-up examinations, including thoracic and abdominal radiography, were performed every 2 to 3 months during the first year after surgical removal of the tumor. Thereafter, owners were interviewed by telephone every 6 months. Formalin-fixed, paraffin-embedded tissue specimens from 89 malignant and 24 benign mammary gland tumors were evaluated by use of immunohistochemical analysis for the identification of hormone receptors.

Immunohistochemical analysis for identification of hormone receptors—Primary antibodies used in the study have been reported elsewhere20 and included mouse anti-human ERα monoclonal antibody (diluted 1:35) and mouse anti-human PR monoclonal antibody (diluted 1:100). Two commercial secondary antibody kits were used with the ERα monoclonal antibody, and another commercial secondary antibody kit was used with the PR monoclonal antibody.

Positive control samples consisted of normal tissues obtained from the ovary, uterus, and mammary glands. Normal ovarian and uterine tissues were obtained from healthy dogs undergoing OHE at our veterinary hospital. Normal mammary gland tissues were obtained from a location far from the neoplastic tissues in dogs with mammary gland tumors. Negative control samples consisted of tissues incubated with PBS solution in place of the primary antibody.

Tissue sections were cut at a thickness of 4 μm, mounted onto silanized slides, and placed in an oven at 45°C for 1 hour. Slides were deparaffinized by 2 immersions in xylene (5 min/immersion) and then immersed 2 times (3 min/immersion) in 100% ethyl alcohol followed by 2 times in 95% ethyl alcohol. Antigen epitope retrieval was achieved by incubation in epitope retrieval solution (Tris-EDTA [pH, 9.0]) for 40 minutes at 95°C to 99°C. Endogenous peroxidase activ-
ity was reduced by addition of peroxidase-blocking reagent. Subsequent immunochemical procedures were performed in accordance with the instruction manual of the manufacturer.

Criteria for determination of staining proportion and staining intensity were based on a scoring system for immunohistochemical staining of ER and PR established in another study. The scoring system included a PS as an estimation of the proportion of positive-staining tumor cells (1 = PS < 1 in 100; 2 = PS from 1 in 100 to 1 in 10, 3 = PS from 1 in 10 to 1 in 3, 4 = PS from 1 in 3 to 2 in 3, and 5 = PS > 2 in 3) and an intensity score for estimating staining intensity of positive-staining tumor cells (0 = negative, 1 = weakly positive, 2 = mildly positive, and 3 = strongly positive). A total score was then calculated by adding the PS and intensity score. For both ER and PR, a total score ≥ 3 was considered a positive result.

**Statistical analysis**—The Kaplan-Meier method was used to construct survival curves for dogs with mammary gland tumors with differences in expression of hormone receptors. The log-rank test was used to identify factors and expression of hormone receptors associated with survival 1 year after surgical removal of the tumor. The Pearson χ² test was used to detect associations between the characteristics of dogs or tumors and hormone receptors. For all analyses, values of P ≤ 0.05 were considered significant. All analyses were performed with commercial software.

**Results**

**Dogs**—Age of the 113 female dogs at the time of tumor removal ranged from 2 to 15 years (mean ± SD, 9.8 ± 2.7 years; median, 10 years). There were 21 breeds represented in these 113 dogs, with mixed-breed dog (n = 31 [27.4%]) dogs, Maltese (23 [20.4%]), Pomeranian (10 [8.8%]), and Toy Poodle (7 [6.2%]) the 4 breeds most commonly represented. The majority of the dogs

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**Table 1**—Hormone receptor expression in 24 benign mammary gland tumors and 89 malignant mammary gland tumors of dogs.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. of tumors</th>
<th>ER+</th>
<th>No.</th>
<th>%</th>
<th>PR+</th>
<th>No.</th>
<th>%</th>
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<td>21</td>
<td>42.9</td>
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<td>Complex carcinoma</td>
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<td>28</td>
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<tr>
<td>Total</td>
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<td>45</td>
<td>50.6</td>
<td>64</td>
<td>71.9</td>
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*Includes simple and complex adenomas. †Includes carcinosarcoma, osteosarcoma, and fibrosarcoma.

ER+ = Expression of ER, PR+ = Expression of PR.

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**Table 2**—Relationship between hormone receptor expression and features of malignant mammary gland tumors of dogs.

<table>
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<tr>
<th>Variable</th>
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<th>No.</th>
<th>%</th>
<th>ER−</th>
<th>No.</th>
<th>%</th>
<th>PR+</th>
<th>No.</th>
<th>%</th>
<th>PR−</th>
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<td>I, II, or III</td>
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*Score based on the TNM system. †Score based on a histologic grading system. **Significant (P < 0.05) association between the investigated variable and expression of the specific hormone receptor.

ER+ = No expression of ER, PR− = No expression of PR.

See Table 1 for remainder of key.
(72 [63.7%] dogs) had a body weight < 10 kg (< 22 lb). In 18 of 113 (15.9%) dogs, OHE was performed prior to surgical removal of mammary gland tumors; reasons for performing OHE were routine spay in 8 dogs and pyometra in 10 dogs. In addition, OHE was performed at the time of tumor removal in 35 dogs; OHE was performed in 13 of the 35 (37.1%) dogs because of pyometra.

Of the 113 dogs, 89 had malignant mammary gland tumors, including 42 (47.2%) complex carcinomas, 35 (39.3%) simple carcinomas, and 12 (13.5%) sarcomas (9 carcinomas, 2 osteosarcomas, and 1 fibrosarcoma). For the 77 carcinomas (complex and simple carcinomas), 24 (31.2%) were classified as grade I, 40 (51.9%) were classified as grade II, and 13 (16.9%) were classified as grade III by use of histologic grading.

Expression of hormone receptors in normal and neoplastic mammary glands—Analysis was performed to detect hormone receptor expression in malignant mammary gland tumors (Figure 1). Normal ovarian, uterine, and mammary gland tissues were used as positive control samples. Expressions of ERα were in the nuclei of mucosal epithelial cells, stromal fibrocytes, and smooth muscle cells of the uterine horns; granulosa cells and interstitial glandular cells of the ovary; and glandular epithelial cells of normal mammary glands. In neoplastic tissues, staining for ERα expression was identified in the nuclei of glandular epithelial cells but not in the cartilage- or osteoid-like area. Moreover, in several tumors, stromal cells surrounding the tumor lesion had moderate to strong staining. Compared with neoplastic cells that had positive staining of various intensities, there was homogenous immunoreactivity of ERα in the normal mammary glands.

Characteristics of PR expression in positive control tissues were similar to those for ERα expression, except that the stromal cells did not have positive staining results for PR. The staining intensity for PR varied in neoplastic tissues.

Relationship between hormone receptors and characteristics of mammary gland tumors—Detection of ERα and PR expression in benign and malignant mammary gland tumors was summarized (Table 1). Except for some benign mixed tumors, most of the benign mammary gland tumors had positive results when tested for ERα and PR expression. However, percentages of expression of ERαs and PRs varied among types of malignant tumors. Accordingly, 68 (60.2%) and 88 (77.9%) mammary gland tumors of the 113 dogs tested, respectively, had positive results when tested to detect expression of ERα and PR. Expression of ERα and PR was detected proportionately more frequently in benign tumors (24/24 [100%] and 23/24 [95.8%], respectively) than in malignant tumors (45/89 [50.6%] and 64/89 [71.9%], respectively). Univariate analysis by use of a chi-square test revealed that hormone receptor expression was not associated with age, breed, OHE prior to tumor removal, history of pyometra, and number and location of affected mammary glands (data not shown). However, among the 11 dogs that had malignant mammary gland tumors with OHE prior to tumor removal, 3 had ERα expression in tumors; the proportion was lower than in malignant mammary gland tumors of dogs (42/78 [53.8%]) without OHE prior to tumor removal. Similar results were also detected with regard to PR expression in dogs that had malignant mammary gland tumors with (6/11) and without (58/78) OHE prior to tumor removal.

To further evaluate the relationship between hormone receptor expression and characteristics of malignant tumors, univariate analysis by use of a chi-square test was used (Table 2). Dogs with malignant tumors with a low clinical stage (I, II, or III) without metastasis to lymph nodes or distant metastasis were significantly more likely to have a higher percentage of ERα expression. With regard to PR expression, the percentage was significantly higher in malignant tumors < 5 cm in diameter, with a low clinical stage (I, II, or III), without metastasis to lymph nodes, and with a low histologic score for tubule formation or mitotic index.

Figure 2—Kaplan-Meier survival curves in dogs with malignant mammary gland tumors grouped on the basis of expression of ERα (A) and PR (B). Analysis revealed that expression of ERα (ERα+) was not significantly associated with survival 1 year after surgical removal of the tumor (log-rank statistic = 1.66; P = 0.198), whereas expression of PR (PR+) was significantly associated with survival 1 year after surgical removal of the tumor (log-rank statistic = 3.93; P = 0.047). Survival time indicates the interval after surgical removal of the tumor.
Comparison of survival in dogs with malignant mammary gland tumors on the basis of hormone receptor expression—Kaplan-Meier survival analysis and log-rank tests were used to investigate the relationship between hormone receptor expression and 1-year survival rate. Results revealed that only PR expression was significantly associated with 1-year survival in dogs with malignant mammary gland tumors (Figure 2). A higher proportion of dogs survived for 1 year when the mammary gland tumor had positive results for PR expression (52/63 [82.5%]), compared with the proportion that survived for 1 year when the mammary gland tumor had negative results for PR expression (15/24 [62.5%]). Additional comparisons were made of the survival curves with regard to the interaction of various hormone receptor expressions (Figure 3). Dogs with tumors that expressed both ERα and PR were more likely to survive for 1 year after surgical removal of the tumor (35/41 [85.4%]), compared with the likelihood of survival for 1 year in dogs with tumors that expressed ERα but not PR (1/3 [33.3%]); survival did not differ significantly between dogs with tumors that expressed both ERα and PR, compared with dogs with tumors that expressed PR but not ERα (17/22 [77.3%]) or expressed neither ERα nor PR (14/21 [66.7%]).

Discussion

The study reported here was conducted to evaluate the relationship of hormone receptors (ERα and PR) and 1-year survival in dogs after surgical removal of mammary gland tumors. Analysis of the results indicated that expression of ERα or PR was significantly associated with tumor size, clinical stage, and metastasis to lymph nodes or distant metastasis, which was similar to results in another report.6 Furthermore, hormone receptor expression also was associated with the histologic scores of tubule formation and mitotic index. In other studies,6–10,22,23 it has been suggested that hormone receptor expression plays an important role in predicting the biological behavior of breast cancer in humans and mammary gland neoplasia in dogs. However, in the study reported here, analysis of survival curves revealed that only PR expression was significantly associated with postoperative survival in dogs with malignant mammary gland tumors. Furthermore, dogs with malignant mammary gland tumors that had expression of both ERα and PR had a significantly higher survival rate, compared with that for dogs with malignant mammary gland tumors that had expression of ERα but not PR. Results of this study were similar to results reported for humans with breast cancer11–10,24 in which a lack of PR expression was associated with secondary breast cancer and PR expression was a better outcome predictor than ER status alone. These observations strongly suggested that expression of PR could be used as a prognostic factor, especially in dogs with malignant gland tumors with ERα expression.

In dogs with malignant mammary gland tumors, the proportion of tumors with PR expression (64/89 [71.9%]) was higher than the proportion of tumors with ERα expression (45/89 [50.6%]); these results were similar to those in another study in dogs (76% vs 31% for PR and ERα, respectively). However, our findings differed from those in reports15,28 for humans with breast cancer, which had a higher percentage (68.8% to 81%) of ERα than that of PR (54% to 70%). Furthermore, lower percentages of ERα expression were detected in malignant tumors involving metastasis to lymph nodes or distant metastasis. Higher percentages of PR expression were detected in tumors < 5 cm in diameter and tumors with histologic tubule formation and a low mitotic index. These findings may imply that expression of ERα and PR is an indicator of differentiation of neoplastic cells. Similarly, lack of ERα and PR expression has frequently been reported in tumor cells with poor differentiation.27,28 It has also been suggested that neoplastic cells with or without ERα and PR expression could originate from different stem cells.29,30

On the basis of results in other studies,11,31 cells with PR expression should have ERα expression because PR expression is modulated by ERα and ERα expression is an essential factor for inducing PRs. In the study reported here, the reason 23 malignant tumors had expression of PR but not of ERα is unclear. However, such a discrepancy could have been caused by the detection limit of the anti-ERα antibody for ERα or as a result of defective ligand binding for biologically functional ERα variants.24,25

Expression of ERαs and tumor cell growth can be suppressed after an ERM (ie, tamoxifen) binds to ERαs.32 Therefore, this technique has been used in treatments for humans with breast cancer. In women with breast cancers, 51.5% of patients whose tumors had expression of both ER and PR responded to tamoxifen treatment; however, only 16.7% of patients whose tumors had expression of ER but not PR responded to the same treatment.9 Similarly, it was also reported16 that patients receiving adjuvant endocrine treatment had a significant decrease in the rate of relapse (53%) and death (38%) when the tumor had expression of both ER and PR, compared with the results when the tumor had expression of ER but not PR. Therefore, from the aspect of clinical treatment,
PR expression may play an important role in predicting better clinical outcome after clinical treatments in human patients whose tumors have expression of ER. Currently, tamoxifen is not recommended as an adjuvant treatment in dogs because of adverse effects such as pyometra, urogenital infection, and vulvar edema. On the basis of our findings, OHE may be used as an adjuvant treatment because the removal of ovaries that produce estrogen may have benefits similar to those of an ERM in humans.

Expression of ERα and PR was detected significantly more often in benign tumors than in malignant tumors. In malignant tumors, expression of ERα and PR was higher in complex carcinomas than in simple carcinomas and sarcomas. Expression of ERα was also associated with other recognized prognostic factors, such as clinical stage, tumor size, and metastasis status. Furthermore, expression of PR was associated with clinical stage, tumor size, metastasis status, tubule formation, and mitotic index. Of the hormone receptors evaluated, only PR was significantly associated with postoperative survival in dogs with malignant mammary gland tumors. These observations strongly suggested that expression of PR could be used as an important prognostic factor, especially in dogs with malignant mammary gland tumors that have ERα expression.

References