RESEARCH COMMUNICATION

Immunohistochemical Determination of Estrogen and Progesterone Receptors in Canine Mammary Tumors

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Abstract

Mammary gland tumors are by far the most commonly found tumors in domestic dogs. Effective therapeutic procedures with prompt accurate diagnoses are of prime importance for this life threatening neoplasm. Although immunohistochemical methods provide valuable information such as the location and semi-quantitative data of the interested antigens in particular tumors, conventional methods like histopathological diagnosis remain useful and necessary for identification and classification of tumors. In the present study, we combined histopathology with immunohistochemical staining of estrogen receptors (ER) and progesterone receptors (PR) in canine mammary gland tumors. Fifty dogs with primary mammary tumors underwent surgery at the Veterinary Teaching Hospital of Mahidol University during 2005 to 2007. Three of them were diagnosed with precancerous lesions and negatively stained for ER or PR antibody. Twenty one were diagnosed with benign tumors classified as adenomas and benign mixed mammary gland tumors. Nearly 60% of the lesions were negatively stained for ER or PR. PR positively stained, both PR and ER stained and ER stained tumors accounted for 19%, 19% and 5%, respectively. Of the malignant tumors, eighty-six percent were adenocarcinomas and 14% were malignant mixed mammary gland tumors. Nearly 70% were negatively stained for ER or PR, 14% were PR positively stained, 14% were both PR and ER stained and 5% were ER stained. Four dogs had unidentified lesions. In summary, more than half of our benign and malignant canine mammary tumors were negatively stained for ER and PR. This indicates a lack of correlation with estrogen and/or progesterone receptor expression.

Key Words: Canine mammary gland tumors - estrogen receptors - progesterone receptors

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Introduction

Mammary gland stem cells are quiescent and self-renewing. Under hormonal regulation, mammary stem cells differentiate to ductal, alveolar and myoepithelial cells. Mouse's mammary gland does not regress during pre-puberty but maintains a small ductal tree which initiates a rapid growth at the onset of puberty (Richiert et al., 2000). The terminal end bud (TEB) composes of cap cell layer surrounds the body cells. The cap cells can take on a myoepithelial lineage or a luminal epithelial lineage (Woodward et al., 2005). To differentiate between luminal epithelial and myoepithelial lineages, we developed immunohistochemical procedure using antibodies against AE1/AE3, vimentin and p63.

Canine mammary tumors are classified as epithelial cell tumor, mesenchymal tumor and mixed tumor (Morrison, 2002). In our research, we are interested in the correlation between steroid receptors, estrogen receptor and progesterone receptor, and canine mammary gland tumors. Conventional method for identification type of tumor is histopathological study, which requires the expertise in interpreting results. However, this method cannot detect the early stage of canine mammary tumors and has no information about stem cell lineaged of mammary tumors (Bergman, 2003; Novosad, 2003; Gama et al., 2003). Immunohistochemical method is a procedure to visualize and differentiate between tissue components using antigen-antibody complex basis. This technique provides valuable information in terms of the location and semi-quantitative data of the interested antigens in the tissue sections for the determination of tumor type (Veerle, 2005).

ER and PR are members of a family of transcription factors expressing during an estrous cycle in domestic dog and regulated by steroid hormones e.g. estrogen and progesterone. During proestrus, estrogen stays low and gradually increases at the end of this stage. Estrogen begins to fall and progesterone continues to rise in the estrus stage. During diestrus, progesterone maintains in quite high level. In anestrus stage, estrogen and progesterone are very low level. Estrogen and

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progesterone dominate and play roles in the canine estrous cycle at various stages. The important role of estrogen involves the proliferation of mammary ducts system at the puberty has been established so far. Progesterone plays an important role in alveoli development.

Ki-67 is a nuclear non-histone protein expressed in cycling cells during late G1, G2, S-phase and M-phase with a molecular mass of 345 and 395 kD. Expression of Ki-67 disappears rapidly after mitosis. In canine mammary tumors, Ki-67 is used as tumor proliferation marker. Malignant tumors are higher proliferation index than benign tumors and dysplastic conditions. High index of Ki-67 is correlated with metastasis, death from neoplasia, low-disease-free survival rates and low overall survival rates (Peña et al, 1998; Zuccari et al, 2004, Thuroczy et al, 2007).

AE1/AE3 monoclonal antibody is a combination of 2 monoclones, AE1 and AE3, both of which recognize a wide spectrum of cytokeratins that are expressed during epithelial cell differentiation. Compared with Epithelial Membrane Antigen (EMA), AE1/AE3 is more specific and has been widely used for clinical determination of the epithelial origin of malignant cells (Tseng et al., 1982; Delsol et al., 1984; Bussolati et al., 1986; Bonnie, 2002). Vimentin, a member of intermediate filaments, is a part of cytoskeleton in cytoplasmic matrix world. Normal canine mammary tissues, mixed tumors and complex adenoma are immunoreactive to Vimentin in every type of myoepithelial cells and mesenchymal tissues (Tateyama et al, 2001; Rabanal and Else, 1994; Destexhe et al, 1993). Therefore, vimentin can be used as a myoepithelial cells and mesenchymal cells marker.

p63, a p53 homologue, is necessary to maintain an epithelial stem cell population, and is widely expressed in basal cells of several types of multilayered epithelial organs (Gama et al, 2003). Monoclonal antibody against p63 is a sensitive and specific myoepithelial marker in canine mammary tumors (Batistatou et al, 2003).

Materials and Methods

Tissue collection

Fifty tissue samples were obtained from female dogs with primary mammary tumors undergoing surgery at the Veterinary Teaching Hospital of Mahidol University from 2005 to 2007. After mammary tumors were removed, tissues were fixed in 10% buffered formalin and then were paraffin-embedded. Three micrometer paraffin sections were cut and stained with and eosin (H&E) for histopathological examination.

Immunohistochemistry

Tissue sections were identified by avidin-biotin-immunoperoxidase method and then used primary antibodies including ER, PR, Ki-67, p63, AE1/AE3, and vimentin. Anti-ER, anti-PR, anti-Ki67 and anti-p63 antibodies were purchased from Santa Cruz Biotechnology, Inc. The anti-ER, anti-PR and anti-Ki67

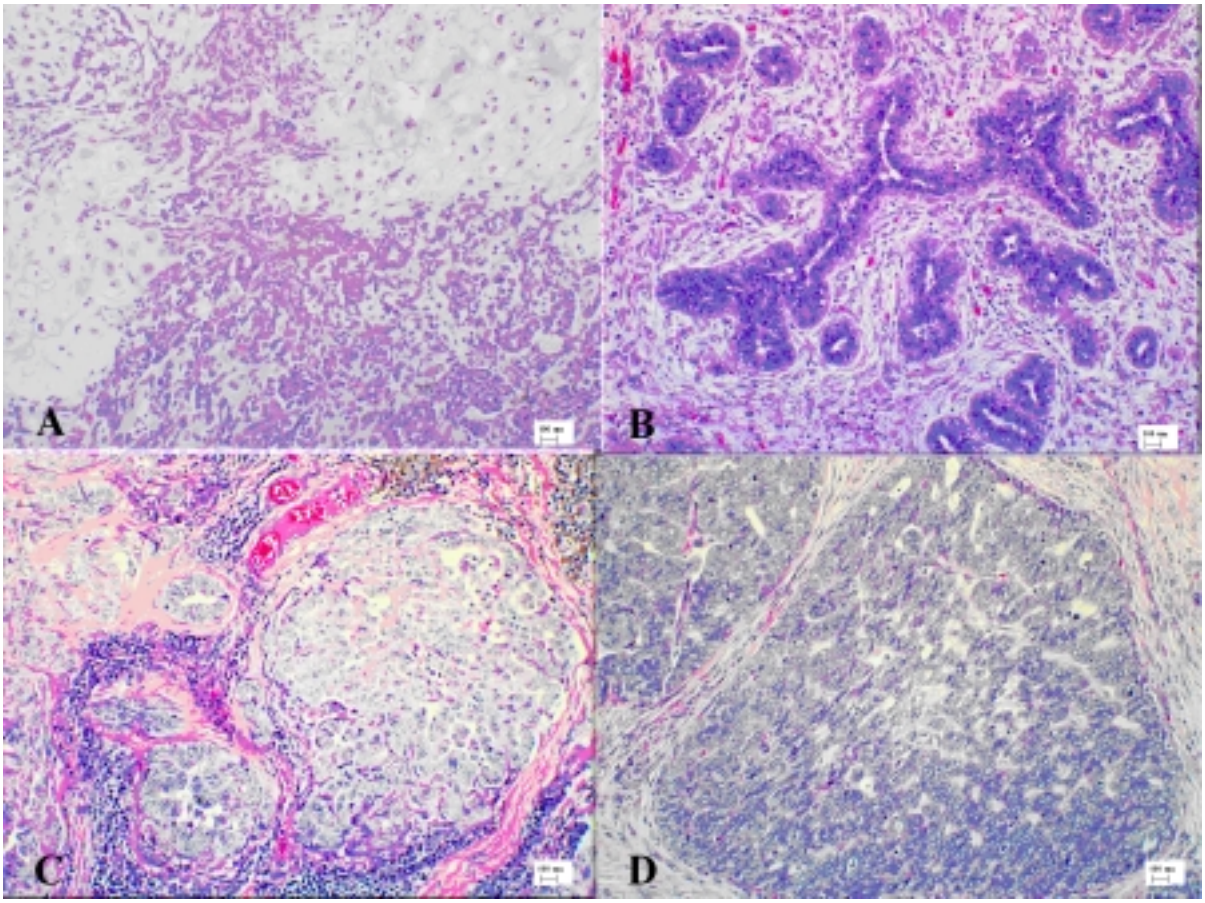

Figure 1. Histopathological Features. A) Acinous adenoma with cartilage metaplasia; B), Tubular adenoma with tubular-like pattern of ductal epithelial cells; C), Papillary tubular carcinoma showing finger-like projections of ductal epithelial cells; and D) Solid carcinoma showed marked mitotic figures. Bars = 100 μm

antibodies were used at 1:50 dilution. The primary antibody against p63 was used at 1:100 dilution. Anti-AE1/AE3 was purchased from Boehringer and used at 1:1000 dilution. Anti-vimentin was purchased from DAKO Corp. and used at 1:300 dilution. Subsequently, the color was developing with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) with H2O2 in sodium acetate buffer. Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted with synthetic resin. Normal canine mammary tissues were used as positive controls. Negative controls were carried out by replacing the primary antibody with PBS.

Evaluation of immunohistochemical data. Positivity was indicated by the presence of distinct dark brown nuclear (ER, PR, p63) or cytoplasmic (AE1/AE3, Vimentin) staining. Classification of staining data was semi-quantitative by using an immunoperoxidase score (Allred et al., 1998).

Results

In a prospective study, 56 mammary gland biopsies were collected at Veterinary Teaching Hospital, Mahidol University, Thailand. Fifty biopsies were classified as primary mammary tumors. Half of them were classified as precancerous lesion, benign mixed mammary gland tumor, tubular or acinous adenoma with precancerous anaplasia, and solid adenoma. Nearly 35% were classified as malignant mixed mammary gland tumor, adenocarcinoma with osseous metaplasia and micrometastasis to lymphatic vessels, tubular carcinoma and solid carcinoma. Fourteen percent were classified as squamous cell carcinoma, osteosarcoma and fibrosarcoma (Table 1).

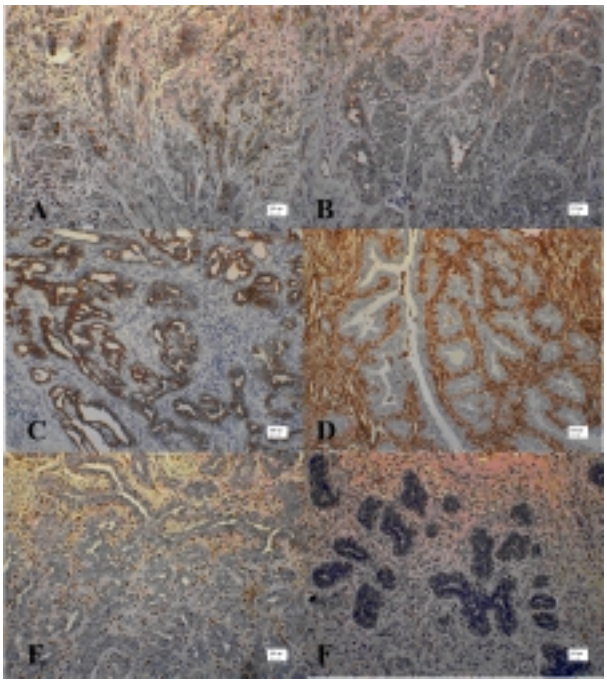

Figure 2. Immunohistochemical Staining. A) Tubular adenoma showing positive dark brown nuclear stained ER; B) Tubular adenoma positive for PR; C) Positive cytoplasmic staining for AE1/AE3; D) Malignant mixed mammary gland tumor positive for cytoplasmic vimentin; E) Positive for p63; F) Tubular adenoma positive for Ki-67. Counterstaining: Harris's hematoxylin. Bars = 100 μm

Table 1. Summary of the Primary Mammary Tumor Diagnoses with Immunoreactivity Data for ER and PR

	Total	Examined*	ER^+	PR^+	ER^+PR^+
Precancerous	1	0	-	-	-
Adenoma	17	14	2	3	1
Benign MT	11	9	3	5	3
Adenocarcinoma	18	14	2	4	2
Sarcoma	3	2	2	1	0
Malignant MT	6	7	1	1	1
Total	56	46	10	14	7

* examined for immunohistochemistry; MT, mixed tumor

According to histopathological studies, the selected mammary tumors were classified as acinous adenoma with cartilage metaplasia, tubular adenoma papillary tubular adenocarcinoma and solid carcinoma (Figure 1). Malignant tumors had a high N:C ratio, marked mitotic figures and pleomorphisms. Fifty canine primary mammary tumors were selected and studied for the correlation between histopathological diagnosis and immunoreactivity to ER, PR, AE1/AE3, Vimentin and p63 data are summarized in Tables 2 and 3 and Figure 2. The selected patients ranged from 6 to 15 years of age. ($X \pm SD = 9.79 \pm 2.18$ years; median = 9.5 years) Twenty one were crossbred and 22 were purebred dogs (8 poodle dogs, 2 Shih Tzu dogs and single representatives of other breeds). For 7 dogs, the breed was not known. Every female dog had masses in the mammary area over a month.

According to AE1/AE3 and vimentin immunopositive, 17 mammary tumors were classified as luminal epithelial origin. Thirteen mammary tumors derived from myoepithelial origin according to immunoreactive vimentin and p63. Sixteen mammary tumors classified as mixed origin were immunopositive for AE1/AE3, vimentin and p63 (Table 3).

In canine mammary glands, AE1/AE3 staining was observed in the cytoplasm of normal epithelial cells, the luminal epithelial and myoepithelial neoplasm as shown in Figure 2C. Vimentin staining was observed in the cytoplasm of normal and tumor of mesenchymal cells including fibrocytes, lipocytes, smooth muscle cells, vascular endothelial cells, astrocytes, Schwann cells, macrophages as well as myoepithelial cells of breast, sweat and salivary glands (Figure 2D). p63 displays a nuclear staining pattern in normal and neoplastic myoepithelial cells but not in ductal, luminal and acinus cells (Figure 2E). Positive staining of Ki-67 is shown in Figure 2F, immunoreactivity being similar among the

Table 3. Summary of Epithelial Cell Lineages

	Luminal	Myoepithelial	Mixed
Adenoma	4	4	6
Adenocarcinoma	6	4	4
Sarcoma	1	1	0
Benign mixed tumor	2	3	4
Malignant mixed tumor	4	1	2
Total/50*	17	13	16

*with 4 unidentified cases

different types of canine primary mammary tumors.

Discussion

In this particular study most mammary tumors commonly arose in the middle-aged and in old dogs (aged an average of 6 to 15 years). Previous study, ER, PR or both receptors positively stained were statistical significant differences among severity and histological subtypes of canine mammary tumors (Martin et al., 2005). According to our study, immunoreactivity to ER or PR had no correlation to tumor types.

There are two epithelial cell types lined the entire normal duct and lobular system of mammary gland, inner luminal cell layer and incomplete outer myoepithelial cell layer. Tumors derived from myoepithelial lineage have been reported as the most frequent canine benign neoplasms. (Moulton, 1990; Foschini and Eusebi, 1998; Lakhani and O'Hare, 2001). Six to fifteen years old female dogs show no differences between number of luminal epithelial, myoepithelial and mixed tumor. However, Luminal epithelial tumors were commonly found in the dogs over 9 years old.

Normally, Ki-67 is expressed in various stages of cell cycle and disappears rapidly after mitosis. Peña et al (1998), Zuccari et al (2004) and Thuroczy et al (2007) revealed that proliferation index of malignant tumors are higher than benign tumors and dysplastic conditions. According to our results, 17 cases were immunoreactivity to Ki-67 and showed no statistical differences between benign and malignant conditions.

According to immunohistochemical studies, it may conclude that canine primary mammary tumors have no correlation to estrogen and/or progesterone receptors expressions. E1/AE3 and Vimentin is a specific marker for identification of canine mammary tumors derived from luminal epithelial lineage. On the other hand, p63 and Vimentin can be used as specific markers for myoepithelial lineaged tumors.

Further observations on survival time and rate, recurrent time and location of recurrent tumor may improve the quality of life of the patient. Stem cell lineage of mammary tumors may provide a huge valuable criterion for selection of the most appropriate therapeutic drug.

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