RESEARCH COMMUNICATION

AE1/AE3, Vimentin and p63 Immunolocalization in Canine Mammary Gland Tumours: Roles in Differentiation between Luminal Epithelial and Myoepithelial Lineages

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Abstract

Mammary tumors are by far the most common tumors in female dogs and effective treatment relies on prompt and accurate diagnostic procedures. Canine mammary tumors may originate from various cell types, such as luminal epithelial, myoepithelial and stromal cells. This study aimed to differentiate luminal epithelial and myoepithelial lineages, using specific markers including AE1/AE3, Vimentin, and p63. Such data can be useful for prognosis. Canine mammary tumors were collected by surgical resection and tissue samples were investigated using the avidin-biotin-immunoperoxidase method with used primary antibodies against AE1/AE3, vimentin, and p63. Luminal epithelial-origin tumors were found to be immunoreactive with AE1/AE3 and vimentin monoclonal antibody, while myoepithelial-origin tumors were positive for p63 and vimentin . In addition, canine mixed tumors showed reactivity with all three antibodies. In summary, AE1/AE3, p63 and vimentin can be used as specific immunohistochemical markers to distinguish lumino-epithelial and myoepithelial lineages of canine mammary tumors.

Key Words: Canine mammary tumors - myoepithelial - luminal epithelial - marker immunohistochemistry

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Introduction

Dogs have the highest incidence of mammary tumors among any mammals, including humans, and the lesions are known for their biologic and histomorphologic heterogeneity. Canine mammary tumors are classified as epithelial, mesenchymal and mixed neoplasms (Morrison, 2002). In our research, we have focused on specific cell type's origin using immunohistochemistry to supply semi-quantitative data for determination of tumor type in the tissue sections of interest (Veerle, 2005).

The 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 other epithelial markers, such as the epithelial membrane antigen (EMA), AE1/AE3 is more specific and has been widely used for clinical determination of the epithelial origin of malignant cells (Bussolati et al, 1986; Bonnie, 2002). Vimentin is a member of intermediate filaments which are parts of cytoskeleton in cytoplasmic matrix world. Normal canine mammary tissues, mixed tumors or complex adenoma are immunopositive to vimentin in every type of myoepithelial cells and mesenchymal tissues (Destexhe et al, 1993; Rabanal and Else, 1994). Therefore, vimentin can be used as a myoepithelial cells and mesenchymal cells markers.p63, a recently characterized p53 homologue, is necessary to maintain an epithelial stem cell population, and it is consistency expressed in basal cells of several types of multilayered epithelial organs (Gama et al., 2003). p63 is a sensitive and specific myoepithelial markers in canine mammary tumors (Batistatou et al., 2003).

Conventional methods utilized for identification of tumor types are histopathological, but this is most appropriate for lesions with thesame stem cell linage (Gama et al., 2003; Novosad, 2003). In our research, we aimed to develop an immunohistochemical method to distinguish between canine mammary tumors of myoepithelial and lumino-epithelial cells lineage using AE1/AE3, vimentin and p63 as markers.

Materials and Methods

Tissue preparation and immunohistochemistry

Fifty tissue samples were obtained from female dogs with primary mammary tumors. All the dogs underwent surgery at the Veterinary Teaching Hospital of Mahidol University among the time of 2005 to 2007.Tissues were fixed with 10% neutral formalin then paraffin-embedded.

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Three micrometer paraffin sections were then cut and routinely stained with hematoxylin and eosin (H&E) for histopathological examination. Tissue sections were deparaffinized, rehydrated and then antigen retrieval was carried out by using microwave heat for 5 minutes at medium voltage in a 10mM citrate buffer, pH 6.0. After cooling at room temperature, the sections were immersed in 3% hydrogen peroxide (H2O2) for 45 minutes and then washed by phosphate buffer saline (PBS), pH 7.6, 3 times. Nonspecific staining was eliminated by 20-minutes incubation with 0.025% normal horse serum (Santa Cruz Biotechnology, Inc). Excess normal serum was removed and replaced by primary antibody (either AE1/AE3, vimentin, p63) and incubated for an hour in a moisture chamber at room temperature following by the incubation at 4 ˚C overnight. After washing the slides, the sections were incubated with a 1:50 dilution of biotinylated secondary antibody followed by avidin-biotin complex (ABC mouse staining system) for 45 minutes. The reagents were purchased from Santa Cruz Biotechnology, Inc. Primary antibody, biotinylated secondary antibody and ABC reagents were diluted in PBS.

The primary antibody AE1/AE3 (monoclonal mouse anti- AE1/AE3, Boehringer) was used at 1:1000 dilution, Vimentin (monoclonal mouse anti-vimentin, DAKO Corp.) were used at 1:300 dilution and p63 (monoclonal mouse anti-p63, Santa Cruz Biotechnology, Inc.) were used at 1:100 dilution. Subsequently, the color was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) with H_2O_2 in sodium acetate buffer for 5 minutes. 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 (p63) or cytoplasmic (AE1/AE3, Vimentin) staining. Classification of staining data was semiquantitative by using an immunoperoxidase score (Allred et al, 1998).

Results

Fifty female dogs ranged from six to fifteen years of age (X ± SD = 9.79±2.18 years; median = 9.5 years). Twenty one were crossbred and twenty-two were purebred dogs (8 poodle breed, 2 Shih Tzu breed and single representatives of other breeds). For seven dogs, breed was not known. Every female dog had masses in mammary area over for 1 month.

According to histopathological study, three patients showed precancerous lesion which classified as

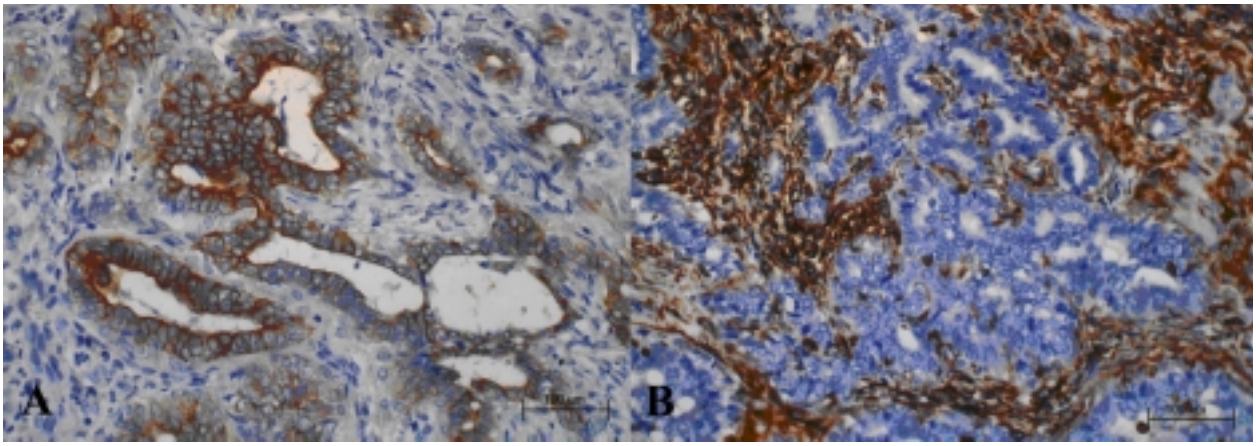

Figure 1. Immunohistochemical Staining of Luminal Epithelial Tumors. Note dark brown color of positive staining of AE1/AE3 (A) in cytoplasm and vimentin (B) in the mesenchymal tissue. Counterstaining: Harris's hematoxylin

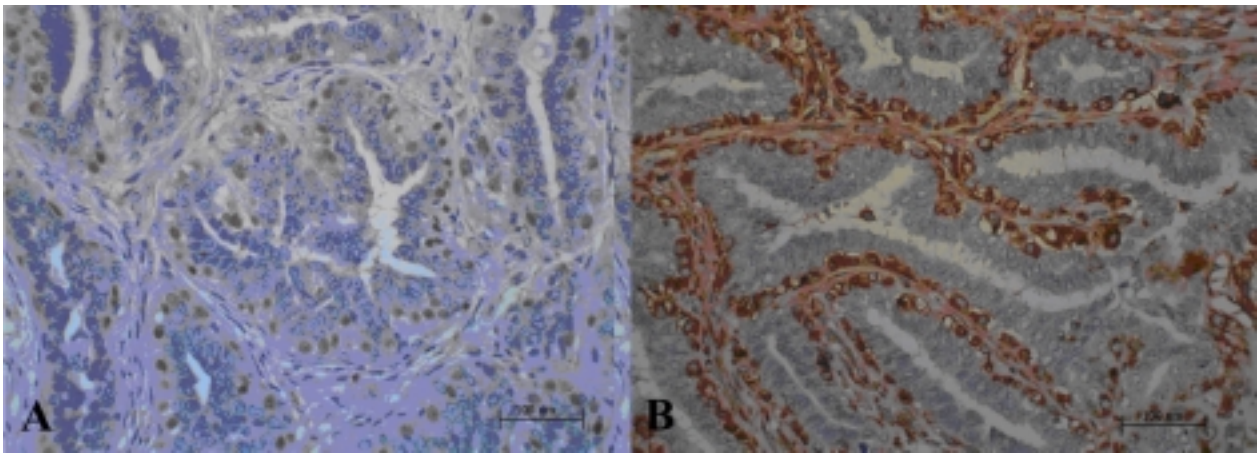

Figure 2. Immunohistochemical Staining of Myoepithelial Tumors. Note dark brown positive staining of p63 (A) in nuclei and cytoplasmic staining of vimentin in myoepithelial cells and mesenchymal cells (B). Counterstaining: Harris's hematoxylin

neuroendocrine-like tumor and hyperplastic condition. Twenty one patients were diagnosis with benign tumor classified as adenoma and benign mixed mammary gland tumor. Interestingly, twenty-two patients were diagnosed as malignant tumor and two of them died in 2007. However, four mammary tissues were unidentified lesions.

In the canine mammary gland, AE1/AE3 staining was observed in the cytoplasm of normal epithelial cells, the luminal epithelial and myoepithelial neoplasm as shown in Figure 1(A) . Vimentin staining was observed in the cytoplasm of normal and mesenchymal cells of tumor, including fibrocytes, lipocytes, smooth muscle cells, vascular endothelial cells, astrocytes, Schwann cells, macrophages as well as myoepithelial cells of breast, sweat and salivary glands. Positive staining of Vimentin was shown in Figure 1(B), 2(B). However, p63 was displayed by a nuclear staining pattern in normal and neoplastic myoepithelial cells whereas ductal, luminal and acinar cells proved completely negative for p63 staining. Positive staining of p63 is shown in Figure 2 (A).

Discussion

In previous study, p63 gene is expressed in the basal cells of several organs, including myoepithelial origin of mammary gland tumor. It is highly specific since neither stormal fibroblast nor vascular smooth muscle cells are not stained (Gama et al, 2003; Veerle, 2005). Vimentin is a 57 kDa intermediated filament (IF) protein, which forms part of the cytoskeleton of vertebrate cells and is important as diagnostic markers in histogenesis of tumors cells (Veerle, 2005). AE1/AE3 mAB, a combination of two colnes, is a cytokeratin that form the cytoskeleton of epithelial cells and presents on most of epithelial neoplasm (Bonnie, 2002). As it has been reported previously, p63 antigen displays a nuclear staining pattern. While vimentin and AE1/AE3 antigen display a cytoplasmic staining pattern. The positive results would show brown-stained (Veerle, 2005; Gama et al, 2003; Gärtner et al, 1999).
Tumor cells with myoepithelial origins are positive for p63 and Vimentin staining. Twenty-eight percent of the canine primary tumors are myoepithelial lineages. One patient had recurrent and metastasis after mass was removed. Six of them had found metastasis.

Luminal epithelial originated tumors were positive for both AE1/AE3 and vimentin staining but negative for p63. Nineteen cases showed luminal epithelial origins. Three female dogs had recurrent and 7 female dogs found metastasis. We demonstrated that mixed tumors were positive for all markers included p63, AE1/AE3 and vimentin staining. Seventeen from 50 mammary masses were originated from both lumino- and myoepithelial cells.

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 showing myoepithelial differentiation have been reported to be the most frequent canine benign neoplasm (Moulton, 1990; Foschini and Eusebi, 1998; Lakhani and O'Hare, 2001). Previous study to distinguish myoepithelail cells by using immunohistochemical markers is based on their dual epithelial and smooth muscle characteristics using high-molecular-weight cytokeratins and smooth muscle actin (Destexhe et al, 1993; Gärtner et al, 1999; Griffey et al, 1993; Vos et al, 1993 a; 1993b; 1993c; Espinosa De Los Monteros et al, 2002). Nevertheless, Gama et al (2003) revealed in their study that p63 is a sensitive and highly specific marker for myoepithelial cells in canine mammary tissues for defining myoepithelial histogenesis. AE1/AE3 and vimentin are also good markers for both luminal and basal epithelial cells in normal mammary gland and mammary tumor (Griffey et al, 1993; Gärtner et al, 1999; Zuccari et al, 2002; Vos et al, 1993a; 1993b; 1993c).
The incidence of age-related occurrence from this study is the most mammary tumors commonly arise in the middle-aged and old dog (average 6 to 15 years). Six to fifteen years old female dogs show no differences in the number of luminal epithelial, myoepithelial and mixed tumors. However, luminal epithelial tumors were commonly found in dogs over 9 years old.

According to the results in this study, we also found that luminal epithelial tumors show worse prognosis than myoepithelial tumors because of the high incidence of recurrence rate after mass resection. Even though a combination of surgery and chemotherapy is a standard for treating mammary tumors in dogs (Novasad, 2003), a large prospective study comparing simple mastectomy to radical mastectomy showed that there was no difference in recurrence rates or survival times (MacEwan et al, 1985; Stratmann et al, 2008). In dogs, the best surgical plan involves removing all of the affected tissue with wide surgical margins (Rutterman et al, 2001).

Doxorubicin has been shown to have some efficacy against canine mammary tumor cell lines in in vitro models (Sartin et al, 1993) while VAC protocol is the optional choice (Sorenmo, 2003). Moreover, mammary tumors can be prevented by spaying the bitches before 6 months of age. The risk of developing a mammary tumor is 0.5% in dogs spayed before 6 months of age or their first heat compared to 26% (and up to 71% in some reports) if spayed after 2 years of age (Schneider et al, 1969). In the study by Sorenmo et al (2000) dogs that were spayed less than two years before mammary tumor surgery can survive longer compared to the group of intact dogs and dogs that were spayed >2 years before mammary tumor treatment.

In conclusion, our research revealed that p63 and AE1/AE3 are specific markers for segregating epithelial mammary tumors from those of myoepithelial lineages. Vimentin and AE1/AE3 can be used to evaluate luminal epithelial tumors. Mixed tumors were positive to AE1/AE3, Vimentin and p63. These markers are new prognostic tools to identify histogenesis of canine mammary tumors.

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