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Immunohistochemical Studies on Cytokeratin 8 and 18 Expressions in Canine Cutaneous Adnexus and Their Tumors

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ABSTRACT. The expressions of cytokeratin 8 and 18 (CK8 and CK18) in the normal canine skin (2 cases) and cutaneous adnexal tumors (127 cases) were investigated immunohistochemically. In the normal skin, co-expression of CK8/18 was found in the glandular epithelium of apocrine sweat glands, and single CK8-immunoreactivity was detected occasionally in the external root sheath at the isthmus and suprabulbar regions of the hair follicles. Neoplastic glandular epithelial cells in all apocrine gland tumors (21/21 cases, 100%) had co-expression of CK8/18. In trichoblastomas (27/28 cases, 96%), most neoplastic cells were diffusely positive for CK8, but those were negative for CK18. Single CK8-expression was also observed in basaloid neoplastic cells in several cases of trichoepitheliomas (7/19 cases, 37%) and pilomatricoma (1/7 cases, 14%). In several cases of trichoblastomas (4/28 cases, 14%) and trichoepitheliomas (2/19 cases, 11%), tumor cells forming glandular structures had co-expression of CK8/18. There were no positive reactions for both CK8 and 18 in infundibular keratinizing acanthomas, and sebaceous and hepatoid gland tumors. The present findings indicate that co-expression of CK8/18 is a specific feature of apocrine sweat glands and single CK8-expression represents the natures of external root sheath or pluripotential stem cells. Thus, the combination of CK8- and 18-immunostainings may have the utility to confirm the directions of differentiation in canine cutaneous adnexal tumors providing a reliable hallmark for histopathological diagnoses.

KEY WORDS: adnexal tumors, canine, cytokeratin 8, cytokeratin 18.

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Adnexal neoplasms of the skin are very common in dogs, whereas those are relatively rare in humans. Recently, World Health Organization (WHO) [2] classifies canine tumors with adnexal differentiations to follicular tumors, nailbed tumors, sebaceous and modified sebaceous gland tumors, apocrine and modified apocrine gland tumors, and eccrine (atrichial) tumors. The new classification in 1998 was estimated considering the direction of differentiation of neoplastic cells.

The development of adnexa is result of an intimate interaction between basal and mesenchymal cells. Basal cells become the germinative cells of the hair follicle and mecenchymal cells become follicular papilla. Follicular stem cells are predominantly considered to be located in the specific region of the external root sheath, especially the bulge region, and are capable to give rise to the hair follicles, epidermis, sebaceous glands, and apocrine sweat glands [4]. Trichoblastoma is considered as benign tumor of hair germ cells. Most cases of basal cell tumors previously diagnosed in the domestic animals have been reclassified into trichoblastomas.

Adnexal neoplasms sometimes have complex histological appearances because of their origin of the pluripotent stem cells. In human, several cases of trichoblastic tumors including trichoblastomas and trichoepitheliomas with complex adnexal differentiations such as apocrine sweat or sebaceous glands were reported [1, 5, 11, 13, 16–18, 20, 23, 26]. Canine adnexal tumors also have similar complex morphological natures, sometimes resulting in confused pathological diagnoses. To determine the useful diagnostic markers and evaluate the directions of differentiation in cutaneous adnexal tumors, several immunohistochemical analyses including cytokeratins (CKs) have been documented in humans [7, 15, 24, 25]. CK8 and 18 are the intermediate filaments of single layer epithelium. CK8 and 18 were generally co-expressed in the glandular epithelium, and used to detect all adenocarcinomas, urinary bladder carcinoma, or hepatocellular carcinoma. Thus, the antibody CAM5•2 capable to detect both CK8 and 18 has been commonly employed. The expression of CAM5•2 (CK8/18) was also demonstrated in the external root sheath of the adult hair follicles in humans [7, 24]. There are also several reports concerning with CKs-expression including CK8 and 18 in normal canine skin and cutaneous tumors [6, 21, 22]. However, most previous studies were performed before the revision of WHO classification, and employed an antibody CAM 5•2 to detect both CK8 and 18.

In the present study, the distributions of CK8 and 18 in normal canine skin and cutaneous adnexal tumors were investigated using each monoclonal antibody (MAb). The utility of the combination of CK8- and 18-immunostainigs to confirm the directions of differentiation in canine cutaneous adnexal tumors providing a reliable hallmark for histopathological diagnoses is discussed.

MATERIALS AND METHODS

Tissue samples: Samples were obtained from biopsy cases between 2002 and 2004 at the Department of Veterinary Pathology, Miyazaki University. Surgical specimens

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from 127 canine adnexal tumors and normal skin tissues of 2 necropsied dogs were collected for immunohistochemistry.

Histopathology: Tissue samples were fixed in 10% formalin. Paraffin sections of 4 μm thickness were stained by hematoxylin and eosin (HE). All cases were diagnosed by general histopathologic examinations according to recent WHO classification [2].

Classification of tumors: Totally, 127 cases of canine adnexal neoplasms were reclassified into infundibular keratinizing acanthomas (10 cases), trichoblastomas (28 cases), trichoepitheliomas (19 cases), pilomatricomas (7 cases), sebaceous gland adenomas (5 cases), sebaceous epitheliomas (11 cases), sebaceous carcinoma (1 case), hepatoid adenomas (10 cases), hepatoid epitheliomas (5 cases), hepatoid carcinomas (10 cases), apocrine adenomas (4 cases), complex apocrine adenomas (3 cases), apocrine carcinomas (13 cases), and complex apocrine carcinoma (1 case) according to the recent WHO classification. The diagnoses of cases with complicated histological features overlapping several criteria were based on the major morphological lesions of the predominant neoplastic cells.

Immunohistochemistry: The sections for CK8 were pretreated by hydrated autoclave for 5 min at 121°C, and those for CK18 were predigested by 0.1% pronase (DAKO-Japan, Kyoto, Japan) for 15 min at 37°C. Each slide was treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The sections were incubated for 30 min at 37°C with primary antibodies. The primary antibodies were mouse monoclonal antibodies against CK8 (prediluted, Progen, Heidelberg, Germany) and CK18 (prediluted, Progen, Heidelberg, Germany). All sections were incubated with Envision Polymer reagent (ENVISION/HRP, DAKO-Japan, Kyoto, Japan) for 30 min at 37°C. The reaction products were visualized by 3,3'-diaminobenzidine (Sigma, St. Louis, MO, U. S. A.) and stained with Mayer's hematoxylin for counter-stain.

RESULTS

Normal skin: Immunohistochemical findings of the normal canine skins are summarized in Table 1. Both CK8- and 18-expressions were detected in the glandular epithelium of apocrine sweat glands (Fig. 1), but not in myoepithelial cells. In the sebaceous and hepatoid glands, there was no immunoreactivity for CK8 and 18. All epidermal layers were also completely negative for both. Single CK8-expression was found in the outermost layer of the external root sheath at the isthmus and suprabulbar regions of the hair follicles (Fig. 1), and the expression was less prominent compared to that of apocrine sweat glands. In addition, the expression was found in a limited number of hair follicles.

Adnexal tumors: The results of CK8- and 18-immunostainings on cutaneous adnexal tumors are summarized in Table 2.

Follicular tumors: Infundibular keratinizing acanthomas were characterized by the proliferation of basaloid epithelial cells with differentiation to squamous epithelium mimicking normal follicular infundibulum. The central lumen was filled with keratin and lined by squamous cells. In all cases of infundibular keratinizing acanthomas, the neoplastic cells were negative for both CK8 and 18.

Table 1. The results of immunohistochemistry for CK8 and 18 in normal canine skin

Skin structures	Immunoreactivity for[a)]	
	CK8	CK18
Epidermis		
Basal layer	–	–
Prickle cell layer	–	–
Hair follicle		
Internal root sheath	–	–
External root sheath	+[b)]	–
Hair matrix	–	–
Sebaceous gland	–	–
Hepatoid gland	–	–
Apocrine sweat gland		
Glandular epithelial cells	+	+
Myoepithelial cells	–	–

a) –: Negative, +: Positive. b) The positive reaction was occasionally found in the isthmus and suprabulbar regions of the hair follicles.

In trichoblastomas, there were several morphological subtypes including ribbon, trabecular, granular cell, and spindle types, and their histologic appearances sometimes complicated. In most cases of trichoblastomas (27/28 cases, 96%), the neoplastic cells were diffusely positive for CK8, whereas those were negative for CK18. The single CK8-expression was well preserved in any morphological subtypes including ribbon (Fig. 2), trabecular, granular cell (Fig. 3), and spindle cell types. The immunoreactivity for CK8 was relatively less intense than that of normal apocrine sweat glands. In several cases of trichoblastomas (4/28 cases, 14%), co-expression of CK8/18 was detected in a limited number of neoplastic cells forming luminal structures (Fig. 4). A case of trichoblastoma had tricholemmomatous changes composed of islands of tumor cells with eosinophilic or vacuolar cytoplasm and separated by thin fibrous tissues. These morphological features were consistent with those of tricholemmoma. The tricholemmomatous foci contained a few CK8-positive cells, while the typical areas of trichoblastomas consisted of a large number of CK8-positive cells.

Trichoepithelioma was divided into benign and malignant tumors mostly according to the degree of invasion activity or metastasis. The tumor exhibited the differentiation into all three segments of the hair follicle. Matrical differentiation with accumulation of shadow cells in the center of the epithelial islands was occasionally observed. In several cases of trichoepitheliomas (7/19 cases, 37%), single CK8-expression was observed in the foci of basaloid cells (Fig. 5), and co-expression of CK8/18 was also detected in the lesions with glandular structures (2/19 cases, 11%, Fig. 6).

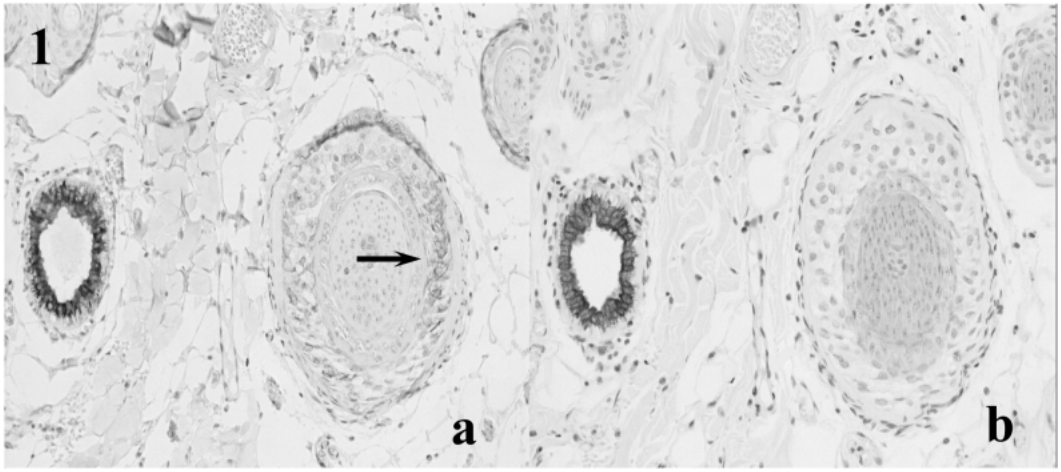

Fig. 1. Normal canine skin. Immunostainings for CK8 (a) and 18 (b). Glandular epithelial cells of the apocrine sweat gland are intensely immunopositive for both CK8 (a) and 18 (b). Outermost layer of the external root sheath at the suprabulbar region of hair follicle has moderate CK8-expression (a, arrow) without that of CK18 (b). × 338.

Table 2. The results of immunohistochemistry for CK 8 and 18 in canine adnexal tumors

Classification of the tumors (n=cases examined)	Number of immunopositive cases for[a)]	
	CK8	CK18
Follicular tumors		
Infundibular keratinizing acanthomas (n=10)	0 (0%)	0 (0%)
Trichoblastomas (n=28)	27 (96%)	4 (14%)
Trichoepitheliomas (n=19)	7 (37%)	2 (11%)
Pilomatricomas (n=7)	1 (14%)	0 (0%)
Sebaceous and modified sebaceous gland tumors		
Sebaceous adenomas (n=5)	0 (0%)	0 (0%)
Sebaceous epitheliomas (n=11)	0 (0%)	0 (0%)
Sebaceous carcinoma (n=1)	0 (0%)	0 (0%)
Hepatoid gland adenomas (n=10)	0 (0%)	0 (0%)
Hepatoid gland epitheliomas (n=5)	0 (0%)	0 (0%)
Hepatoid gland carcinomas (n=10)	0 (0%)	0 (0%)
Apocrine gland tumors		
Apocrine adenomas (n=4)	4 (100%)	4 (100%)
Complex and mixed adenomas (n=3)	3 (100%)	3 (100%)
Apocrine carcinomas (n=13)	13 (100%)	13 (100%)
Complex and mixed carcinoma (n=1)	1 (100%)	1 (100%)

a) The number in the parenthesis represents the percentage of positive cases per examined cases.

Pilomatricoma was characterized by exclusive matrical differentiation, and classified into benign and malignant. The tumors consisted of several lobules separated by collagenous stroma. The periphery of the lobule was composed of the proliferation of basophilic cells. These basophilic cells differentiate toward the center of the lobule with ghost or shadow cells representing matrical differentiation. In a case of pilomatricoma (1/7 cases, 14%), the basophilic cells in the peripheral lobule showed CK8-immunoreactivity without CK18-expression.

Sebaceous and modified sebaceous gland tumors: Sebaceous gland tumors were classified into sebaceous adenomas, epitheliomas, and carcinomas. No immunoreactivities for both CK8 and 18 were detected in all components of sebaceous gland tumors, such as sebocytes and basaloid cells. In a similar manner, there were also no immunoreactivities in all cases of hepatoid gland tumors including hepatoid gland adenomas, epithelioma, and carcinomas.

Apocrine and modified apocrine gland tumors: All apocrine gland tumors including apocrine adenomas, complex and mixed adenomas, carcinomas, and complex and mixed carcinomas were positive for both CK8 and 18 in spite of morphological variation and malignancy (Fig. 7). Two cases of apocrine carcinomas were mainly composed of neoplastic cells with abundant clear cytoplasm and scanty glandular structures. These clear cells were also intensely positive for both CK8 and 18. In complex and mixed tumors, co-expression of CK8/18 was found in the tumor cells forming glandular structures, whereas surrounding

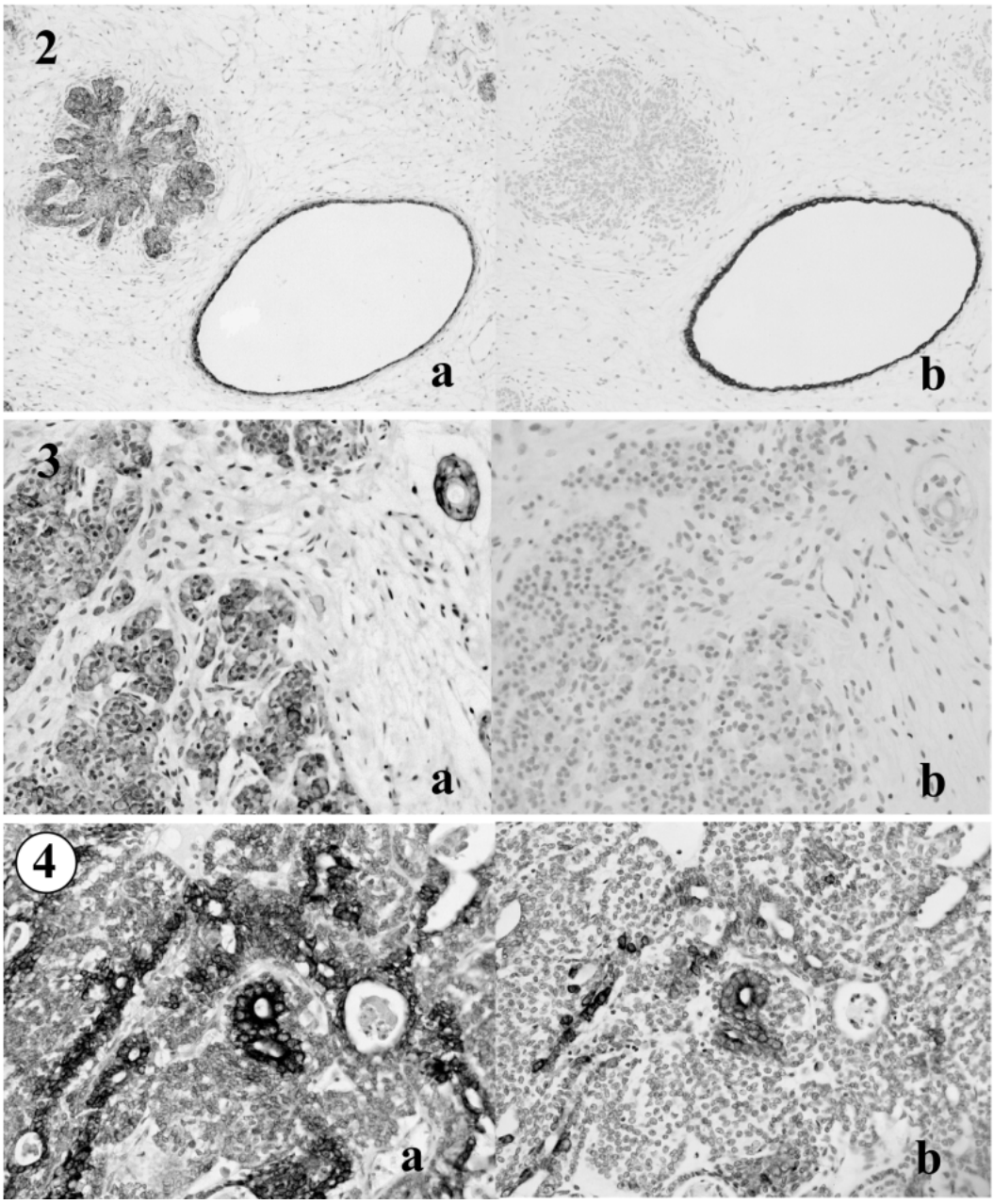

Fig. 2. Trichoblastoma, ribbon type. Immunostainings for CK8 (a) and 18 (b). The tumor cells are positive for CK8 (a), but those are negative for CK18 (b). × 68.

Fig. 3. Trichoblastoma, granular cell type. Immunostainings for CK8 (a) and 18 (b). Tumor cells are diffusely positive for CK8 (a), but are negative for CK18 (b). × 169.

Fig. 4. Trichoblastoma, trabecular type. Immunostainings for CK8 (a) and 18 (b). Tumor cells with glandular structures have co-expression of CK8 (a) and 18 (b). Basaloid tumor cells are positive for CK8, but are negative for CK18. × 169.

αSMA-positive-myoepithelial cells were negative for these CKs. The distribution of CK18-positive cells in apocrine gland tumors was almost consistent with that of CK8. However, the immunoreactivity to CK18 was less prominent compared to that to CK8 in several apocrine carcinomas.

DISCUSSION

There are several documents related to the distributions of CKs in the normal canine epidermis and cutaneous adnexal tumors [6, 21, 22]. However, respective distributions

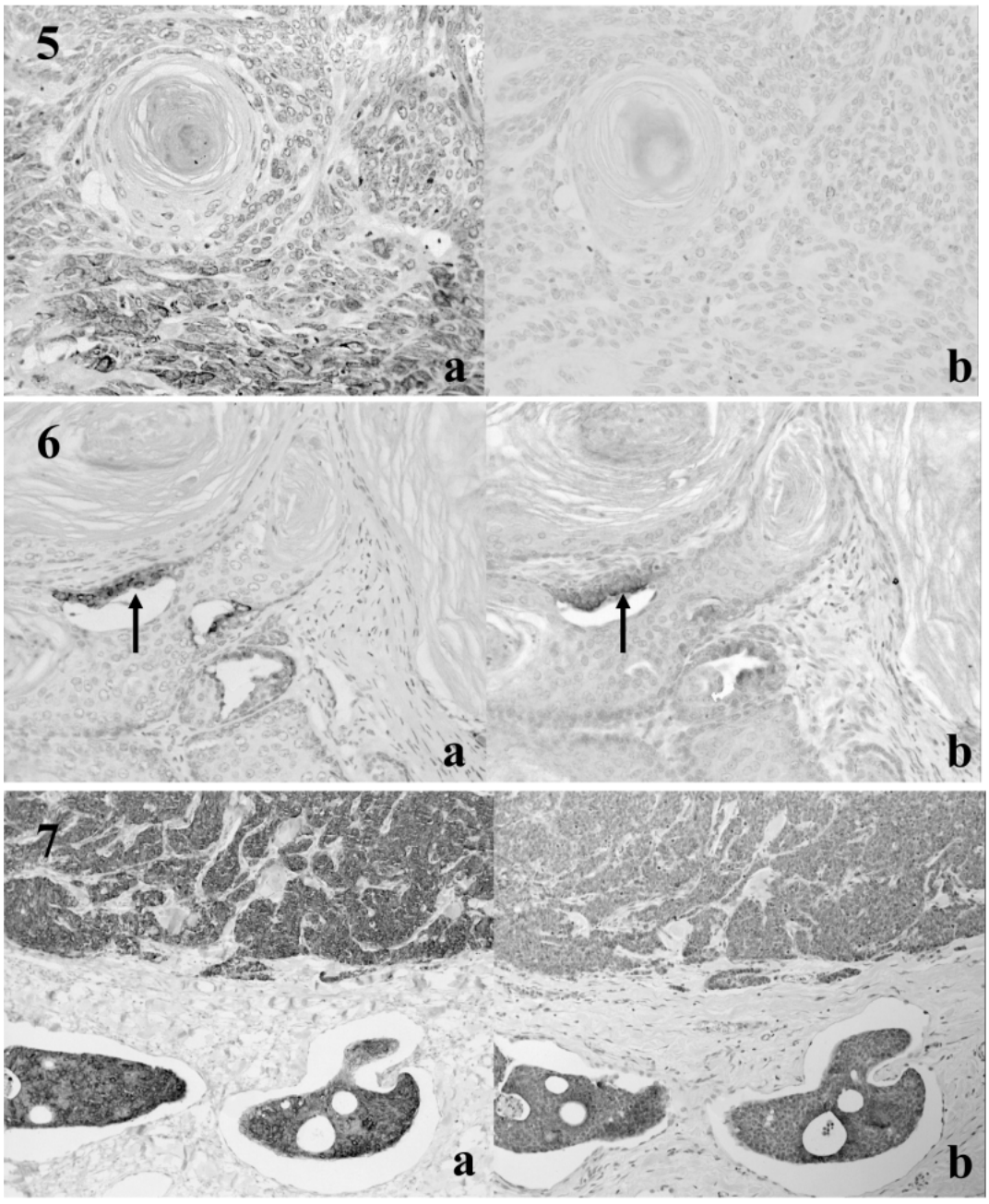

Fig. 5. Trichoepithelioma. Immunostainings for CK8 (a) and 18 (b). Basaloid tumor cells are positive for CK8 (a), but are negative for CK18 (b). × 169.

Fig. 6. Trichoepithelioma. Immunostainings for CK8 (a) and 18 (b). Neoplastic cells forming glandular structures (arrows) have co-expression of CK8 (a) and 18 (b). × 169.

Fig. 7. Apocrine carcinoma. Immunostainings for CK8 (a) and 18 (b). Almost neoplastic cells have co-expression of CK8 (a) and 18 (b). × 68.

of CK8 and 18 in canine cutaneous adnexa and their neoplasms were not well demonstrated because antibody CAM5•2 which recognizes both CK8 and 18 at once, were generally employed. In the present study, the presence of CK8 and 18 were investigated using each MAb. In the normal skin, both CK8- and 18-positive cells were localized in the glandular epithelium of the apocrine sweat glands. Neoplastic epithelial cells in all apocrine gland tumors also had co-expression of CK8/18 despite the morphological variation and malignancy. The findings suggest that CK8/18 co-

expression in the canine cutaneous adnexa could be the specific marker of the glandular epitheliums of apocrine sweat glands. Interestingly, in several trichoblastomas (4/28 cases, 14%) and trichoepitheliomas (2/19 cases, 11%), co-expression of CK8/18 was also found in tumor cells forming glandular structures. Recently, gross cystic disease fluid protein-15 (GCDFP-15) has been recognized as reliable marker of glandular epithelial cells of eccrine and apocrine sweat glands in humans [17]. Further investigations using such antibody will confirm the differentiation of several canine follicular tumors into apocrine glands.

Single CK8-expression was found in the outermost layer of the external root sheath of the isthmus and suprabulbar regions of the hair follicles in the normal canine skin, whereas all other follicular components were negative. Therefore, single CK8-expression might closely associate with the external root sheath of these regions. Since CK8-positive cells were not always found in all hair follicles, the single CK8-expression might depend on hair cycle. In almost cases of trichoblastomas (27/28 cases, 96%), the majority of neoplastic cells also showed single CK8-expression. Similar CK8-expression was occasionally observed in trichoepitheliomas and pilomatricoma. Multiple differentiations towards more than one type of adnexal structures including hair follicles and apocrine sweat glands were observed in several trichoblastomas and trichoepitheliomas. Furthermore, histological and immunohistochemical findings indicate the close relationship between trichoblastomas, trichoepitheliomas, pilomatricomas, and apocrine gland tumors. The findings support the concept that trichoblastoma is derived from the follicular germinative cells [2], and these adnexal neoplasms are originated from common stem cells. Moreover, single CK8-expression might associate with not only external root sheath but also pluripotent stem cells population. In a case of trichoblastoma with tricholemmomatous changes, the foci contained only a few CK8-positive cells unlike typical lesions of trichoblastomas. The finding indicates the single CK8-expression might decrease according to the differentiations into mature follicular components. Follicular tumors negative for CK8 might reflect well-differentiated natures of these tumors. In human, immunoreactivity to CK19 has been detected in the adult and fetal hair follicles [4, 7–9, 24], and the expressions are considered to reflect the site of the pluripotential stem cells. Since CAM 5•2 (CK8/18) expression in the hair follicles is overlapping with the distribution of CK19-positive cells, these CKs also have some associations with the stem cells of the cutaneous adnexa. Although CK19 could be applied to canine tissues, the expression was detected in normal glandular epithelium of apocrine sweat glands [21] and myoepithelial cells of mammary glands [19]. Thus, single CK8-expression in the canine cutaneous adnexa might be more useful hallmark of follicular germinative cells than CK19. Further investigation on canine fetal skin tissue will provide additional information to determine a keratin-phenotype of follicular stem cells.

On the contrary, no immunoreactivities for both CK8 and 18 were detected in all cases of infundibular keratinizing acanthomas, and sebaceous and hepatoid gland tumors. Canine sebaceous epitheliomas had some histological overlapping features with infundibular keratinizing acanthomas and the phenomenon might reflect the interaction between normal sebaceous ducts and their opening portion of infundibulm. In human, these tumors are also supposed to be derived from a common pluripotent stem cell of the hair follicle because of the presence of adenxal tumors with multiple differentiations such as trichoblastoma with sebaceous gland differentiation [1, 26] or sebaceous carcinoma with apocrine sweat gland differentiation [11, 12, 16]. However, the present data indicate that canine infundibular keratinizing acanthomas and sebaceous and hepatoid gland tumors might complete specific differentiation and lose CK8-expression.

Among canine cutaneous adnexal neoplasms, clear cell appearances of the tumor cells were occasionally recognized. Mikaelian *et al.* [10] proposed a new concept of canine follicular tumor as follicular stem cell carcinoma characterized by clear cells with apocrine or trichoepitheriomatous adnexal differentiation. On the other hand, Nibe *et al.* [14] reported apocrine adenoma clear cell variant. The previous paper also demonstrated co-expression of CK8 and 18 in this tumor, and utility of these CKs and αSMA for apocrine sweat glands tumors. In the present study, two cases of apocrine carcinomas were mainly composed of neoplastic cells with abundant clear cytoplasm and scanty glandular structures. The morphology of these cases was quite similar to clear-cell hidradenocarcinomas [3] or apocrine adenoma clear cell variant [14] previously described in dogs. These clear cells in our cases also showed co-expression of CK8 and 18, suggesting the apocrine sweat gland origin of the neoplastic cells. Thus, the combination of CK8- and 18-immunostainings is the useful utility to investigate directions of differentiation in adnexal tumors with various morphological features.

In conclusion, the paper reveals that co-expression of CK8/18 is a specific feature of canine glandular tissues including apocrine sweat glands and single CK8-expression without CK18 is characteristic for the external root sheath or pluripotential follicular stem cells. Thus, the combination of CK8- and 18-immunostainings may have the utility to confirm the directions of differentiation in canine cutaneous adnexal tumors providing a reliable hallmark for histopathological diagnoses.

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