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Immunohistochemical Analysis of Cyclin A, Cyclin D1 and P53 in Mammary Tumors, Squamous Cell Carcinomas and Basal Cell Tumors of Dogs and Cats

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ABSTRACT. The involvement of cyclin A, cyclin D1 and p53 proteins in canine and feline tumorigenesis was analyzed immunohistochemically. In the present study, a total of 176 cases were examined, among which there were 108 canine cases (75 mammary lesions, 16 squamous cell carcinomas and 17 basal cell tumors) and 68 feline cases (43 mammary lesions, 20 squamous cell carcinomas and 5 basal cell tumors). Speckled nuclear staining for cyclin A was observed in 19/38 (50%) canine malignant mammary tumors and 18/37 (48.6%) feline mammary carcinomas, while this was not seen in benign mammary tumors of either dogs or cats. Marked intense nuclear cyclin A staining was seen in 7/16 (43.8%) canine squamous cell carcinomas and 18/20 (90.0%) feline squamous cell carcinomas. Only 3/17 (17.6%) canine basal cell tumors showed slight and scattered staining for cyclin A. Expression of cyclin D1 was very rare in both canine and feline tumors. Nuclear staining of p53 was found in 7/37 (18.9%) feline mammary carcinomas. Intense immunoreactivity for p53 was found in 6/16 (37.5%) canine squamous cell carcinomas and 8/20 (40%) feline squamous cell carcinomas. These results suggest that cyclin A may have a role in the proliferation of canine malignant mammary tumors, feline mammary carcinomas and squamous cell carcinomas of dogs and cats, and p53 may associate with the tumorigenesis of feline mammary carcinomas and squamous cell carcinomas of dogs and cats.

KEY WORDS: canine, cyclin A, cyclin D1, feline, p53.

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The critical role that the family of regulatory proteins known as cyclins play in eukaryotic cell cycle regulation is well established. Cyclins are categorized into three types; A-type, B-type, G1 cyclins (C-, D-, and E-types), that act by forming a complex with cyclin dependent kinases (cdk) at various stages of the cell cycle. Phosphorylation of the retinoblastoma protein by these complexes leads to the release of a variety of transcription factors, usually represented by E2F family members, and is considered to be the major driving event in the transition from G1 to S phase of the cell cycle. Altered expression of several cyclins in human cancer has been recognized in the past few years.

Cyclin A, a protein of 60 kDa, binds independently to cdk2 in S to G2 phase, and cdk2/cdc2 in G2 to M phase, leading to enzyme activation. Cyclin A is detectable in S phase, and increases during cell cycle progression to G2 phase. Cyclin A is overexpressed in some hepatocellular carcinomas because it lacks a cyclin destruction box due to genomic insertion by the hepatitis B virus [7]. Cyclin A alterations have also recently been identified in several tumors including squamous cell carcinomas of the lung [9, 44–46], oral cavity [22], esophagus [11] and uterine cervix [20].

The D-type cyclins act primarily by regulating the activity of cdk4/cdk6 in the G1 phase of the cell cycle. In human, cyclin D1, also known as PRAD-1 or bcl-1, is a 36 kDa protein and the cyclin D1 gene is located on 11q13. Maximum expression of cyclin D1 occurs at a critical point in mid to late G1 phase. Overexpression of cyclin D1 has been found in a wide variety of human tumors, such as breast cancers [1, 3, 8, 13, 21, 24, 30, 39, 48], head and neck cancers [4, 26] and esophageal cancers [18,19, 32], sometimes due to gene amplification.

In human, the p53 tumor suppressor gene is located on the short arm of chromosome 17 and its protein product is a negative regulator of the cell cycle in the G1 phase. Mutations in the p53 gene are the most frequent alteration in many types of human malignancy, including lung, colon and breast cancers. Recently, cDNAs for canine and feline p53 have been molecularly cloned [29, 43]. P53 mutation has been reported in canine mammary tumors [23], canine osteosarcomas [25, 42] and feline hematopoietic tumors [29], and besides the protein overexpression has been reported in several tumors, such as canine mammary tumors [12, 17, 34] and, canine and feline squamous cell carcinomas [12, 40].

There are few data about alterations of cyclin expression in canine and feline tumors. In this study, immunohistochemical analysis for cyclin A, cyclin D1 and p53 expression in mammary tumors, squamous cell carcinomas and basal cell tumors of dogs and cats was performed in order to clarify whether overexpression of these gene products correlates with canine and feline tumorigenesis.

MATERIALS AND METHODS

Tissue samples: A total of 176 cases were obtained from surgical specimens between 1996 and 1998 at the Department of Veterinary Pathology, Miyazaki University, Japan. For histopathology, the specimens were fixed in 10% neutral buff-

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Table 1. Histological typing of 176 cases examined

Species	Histological type	Number of cases
Dog		108
	Mammary	75
	Adenosis	2
	Benign tumor	35
	Adenoma	25
	Benign mixed tumor	10
	Malignant tumor	38
	Adenocarcinoma	33
	Malignant mixed tumor	3
	Malignant myoepithelioma	2
	Squamous cell carcinoma	16
	Well differentiated type	5
	Common type	5
	Poorly differentiated type	6
	Basal cell tumor	17
Cat		68
	Mammary	43
	Adenosis	4
	Fibroadenoma	2
	Carcinoma	37
	Well differentiated type	10
	Common type	11
	Poorly differentiated type	16
	Squamous cell carcinoma	20
	Well differentiated type	5
	Common type	8
	Poorly differentiated type	5
	Bowen's disease	2
	Basal cell tumor	5
	Total	176

ered formalin, cut at a thickness of 4 μm and stained with hematoxylin and eosin (HE) for light microscopy. The pathologic characteristics of 176 cases are listed in Table 1. One hundred and seventy-six cases comprised 108 canine cases (75 mammary lesions, 16 squamous cell carcinomas and 17 basal cell tumors) and 68 feline cases (43 mammary lesions, 20 squamous cell carcinomas and 5 basal cell tumors). Both canine and feline squamous cell carcinomas were restricted to the cases of origin of skin. The 75 canine mammary lesions were divided into 3 groups comprising 2 adenosis, 35 benign tumors (25 adenomas and 10 benign mixed tumors) and 38 malignant tumors (33 adenocarcinomas, 3 malignant mixed tumors and 2 malignant myoepitheliomas). The 43 feline mammary lesions included 4 adenosis, 2 fibroadenomas, and 37 feline mammary carcinomas. The grade for feline mammary carcinomas was derived from the assessment of three morphological features: degree of tubule formation, degree of nuclear and cellular pleomorphism and mitotic activity. Thirty-seven feline mammary carcinomas were divided into 3 types as 10 well differentiated types, 11 common types and 16 poorly differentiated types. Classification of canine and feline squamous cell carcinomas was based on the morphological features such as degree of cancer pearl formation, degree of nuclear and cellular pleomorphism and mitotic activity. Sixteen canine squamous cell carcinomas were graded as 5 well differentiated types, 5 common types and 6 poorly differentiated types. Twenty feline squamous cell carcinomas were also graded as 5 well differentiated types, 8 common types, 5 poorly differentiated types and 2 cases of Bowen's disease.

Antibody: The primary antibodies used were a rabbit polyclonal antibody against human cyclin A, a recombinant protein corresponding to amino acids 1–432 representing full-length cyclin A of human origin (H-432, Santa Cruz Biotech, U.S.A.), a rabbit polyclonal antibody against human cyclin D1, a recombinant protein corresponding to amino acids 1-295 representing full-length cyclin D1 of human origin (H-295, Santa Cruz Biotech, U.S.A.) and a rabbit polyclonal antibody against human p53 antibody, recognizing the wild-type and mutant forms of p53 (CM1, Novocastra, UK).

Immunohistochemistry: Immunohistochemistry was performed using an Envision polymer reagent (DAKO, Kyoto, Japan). Hydrated autoclave treatment was performed on the paraffin sections before immunostaining. Endogenous peroxidase was quenched by 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The sections were incubated with 3% (w/v) bovine serum albumin in PBS for 1 hr at 37°C. Primary antibodies against cyclin A (1:40), cyclin D1 (1:40) and p53 (1:100) were applied. The sections were incubated with these primary antibodies overnight at 4°C and with Envision polymer reagent for 30 min at 37°C. The chromogenic reaction was carried out with 3,3-diaminobenzidine-4 HCl (0.5 mg/m*l*) in Tris-hydrochloride buffer, pH 7.6, supplemented with 0.03% (v/v) hydrogen peroxide and counterstained with Mayer's hematoxylin.

Microscopic evaluation: The immunoreactivities for cyclin A, cyclin D1 and p53 were assessed using a grading system based on the percentage of positive nuclei. The staining was scored as 0, tumor with no nuclear staining; 1, tumor with <10% of its nuclei stained; 2, tumor with 10–50% of its nuclei stained; 3, tumor with >50% of its nuclei stained. Specimens assigned scores of 2 to 3 were considered positive.

RESULTS

The immunohistochemical data for expression for cyclin A, cyclin D1 and p53 are summarized in Tables 2 and 3, and the details are described below.

Cyclin A: Nuclear staining of cyclin A was observed in 19/75 (25.3%) canine mammary lesions, 18/43 (41.9%) feline mammary lesions, 7/16 (43.8%) canine squamous cell carcinomas, 18/20 (90%) feline squamous cell carcinomas and 3/17 (17.6%) canine basal cell tumors, but was not seen in feline basal cell tumors. Among the malignant mammary tumors, 19/38 (50%) canine malignant tumors and 18/37 (48.6%) feline mammary carcinomas showed moderate to intense and speckled nuclear staining in the glandular epithelium (Fig. 1A). In contrast, adenosis, benign tumors and normal mammary gland showed only rare or weak staining in the epithelium. One canine malignant myoepithelioma also showed intense staining for cyclin A diffusely in the nuclei of the myoepithelium (Fig. 1B), but myoepithelium components of all adenoma cases were negative for cyclin A. Furthermore, 7/16 (43.8%) canine and 18/20 (90%) feline squamous cell carcino-

Table 2. Expression of cyclin A, cyclin D1 and p53 in canine and feline mammary lesions

Species	Histological type	Percentage of positive cases (numbers)		
		cyclin A	cyclin D1	p53
Dog	Mammary	25.3% (19/75)	2.7% (2/75)	ND
	Adenosis	0.0% (0/2)	0.0% (0/2)	ND
	Benign tumor	0.0% (0/35)	0.0% (0/35)	ND
	Adenoma	-	-	ND
	Benign mixed tumor	-	-	ND
	Malignant tumor	50.0% (19/38)	5.3% (2/38)	ND
	Adenocarcinoma	51.5% (17)	6.1% (2)	ND
	Malignant mixed tumor	33.3% (1)	-	ND
	Malignant myoepithelioma	50.0% (1)	-	ND
Cat	Mammary	41.9% (18/43)	0.0% (0/43)	16.3% (7/43)
	Adenosis	0.0% (0/4)	0.0% (0/4)	0.0% (0/4)
	Fibroadenoma	0.0% (0/2)	0.0% (0/2)	0.0% (0/2)
	Carcinoma	48.6% (18/37)	0.0% (0/37)	18.9% (7/37)
	Well differentiated type	40.0% (4)	-	-
	Common type	36.4% (4)	-	18.2% (2)
	Poorly differentiated type	62.5% (10)	-	31.3% (5)

*ND: not done.

Table 3. Expression of cyclin A, cyclin D1 and p53 in squamous cell carcinomas and basal cell tumors of dogs and cats

Species	Histological type	Percentage of positive cases (numbers)		
		cyclin A	cyclin D1	p53
Dog	Squamous cell carcinoma	43.8% (7/16)	6.3% (1/16)	37.5% (6/16)
	Well differentiated type	-	20.0% (1)	40.0% (2)
	Common type	40.0% (2)	-	-
	Poorly differentiated type	83.3% (5)	-	66.7% (4)
	Basal cell tumor	17.6% (3/17)	0.0% (0/17)	0.0% (0/17)
Cat	Squamous cell carcinoma	90.0% (18/20)	0.0% (0/20)	40.0% (8/20)
	Well differentiated type	100.0% (5)	-	40.0% (2)
	Common type	100.0% (8)	-	25.0% (2)
	Poorly differentiated type	100.0% (5)	-	80.0% (4)
	Bowen's disease	-	-	-
	Basal cell tumor	0.0% (0/5)	20.0% (1/5)	0.0% (0/5)

mas had large numbers of cancer cells positive for cyclin A (Figs. 1C and D). Intense nuclear staining was observed predominantly in the peripheral cells of neoplastic foci (Fig. 1C). The proportion of positive cells and the degree of staining intensity varied between tumors. In only 2 canine squamous cell carcinomas, the immunological staining was observed in the nucleus and cytoplasm of neoplastic cells. Feline squamous cell carcinomas exhibited relatively higher immunoreactivity for cyclin A than canine squamous cell carcinomas. Very weak staining was rarely observed in the normal epithelium adjacent to the tumor cells and was always restricted to the basal cell layer. The percentage of poorly differentiated canine squamous cell carcinomas showing positivity for cyclin A was much higher than those of lower grades. All of the feline squamous cell carcinomas showed very strong staining for cyclin A, except for 2 cases of Bowen's disease. The cyclin A positivity of basal cell tumors was obviously lower than that in other tumors. Only 3/17 (17.6%) canine basal cell tumors had scattered positive cells with weak immunoreactivity for cyclin A, and all of the feline basal cell tumors were negative.

Cyclin D1: Among 176 cases, only 4 cases including 2 canine mammary adenocarcinomas, one canine squamous cell carcinoma and one feline basal cell tumor were positive for cyclin D1. These cases exhibited different patterns of staining. Two canine adenocarcinomas showed weak and speckled nuclear staining in the epithelium and the intensity was much lower than that of cyclin A. However, one canine squamous cell carcinoma, which was well differentiated, had markedly strong staining restricted to the peripheral cells of neoplastic foci (Fig. 2A). In one feline basal cell tumor, moderate and focal nuclear staining was observed in the tumor cells, which showed a solid pattern (Fig. 2B), whereas weak and scattered staining was observed partly in the tumor cells showing keratinization.

p53: Nuclear staining of p53 was found in 7/43 (16.3%) feline mammary lesions, 6/16 (37.5%) canine squamous cell carcinomas and 8/20 (40%) feline squamous cell carcinomas,

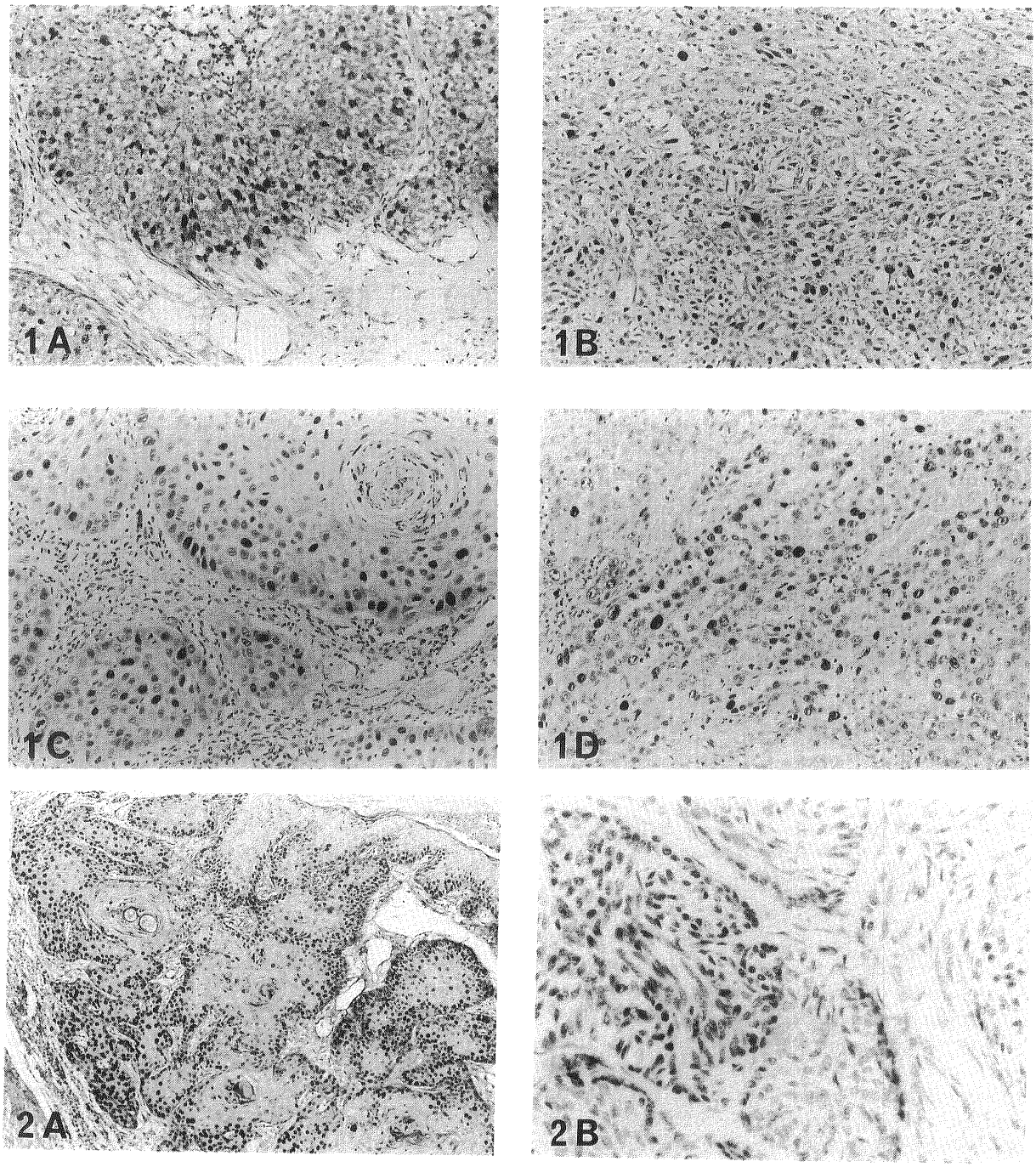

Fig. 1. Immunohistochemistry for cyclin A. (A) Speckled and intense nuclear staining of the glandular epithelium in a canine mammary adenocarcinoma. × 200. (B) Diffusely intense nuclear staining of the myoepithelium in a canine malignant myoepithelioma. × 200. (C) Intense nuclear staining of the peripheral neoplastic cells of tumor island in a canine squamous cell carcinoma (common type). × 200. (D) Intense nuclear staining of the neoplastic squamous epithelium in a feline squamous cell carcinoma (poorly differentiated type). × 200.

Fig. 2. Immunohistochemistry for cyclin D1. (A) Markedly strong staining restricted to the peripheral neoplastic cells of tumor island in a canine squamous cell carcinoma (common type). × 100. (B) Moderate and focal nuclear staining (left side) in a feline basal cell tumor. × 400.

but this was not found both in either canine or feline basal cell tumors. In feline mammary tumors, 7 (2 common types and 5 poorly differentiated types) of 37 (18.9%) feline mammary carcinomas expressed p53 protein in the nuclei of the epithe-

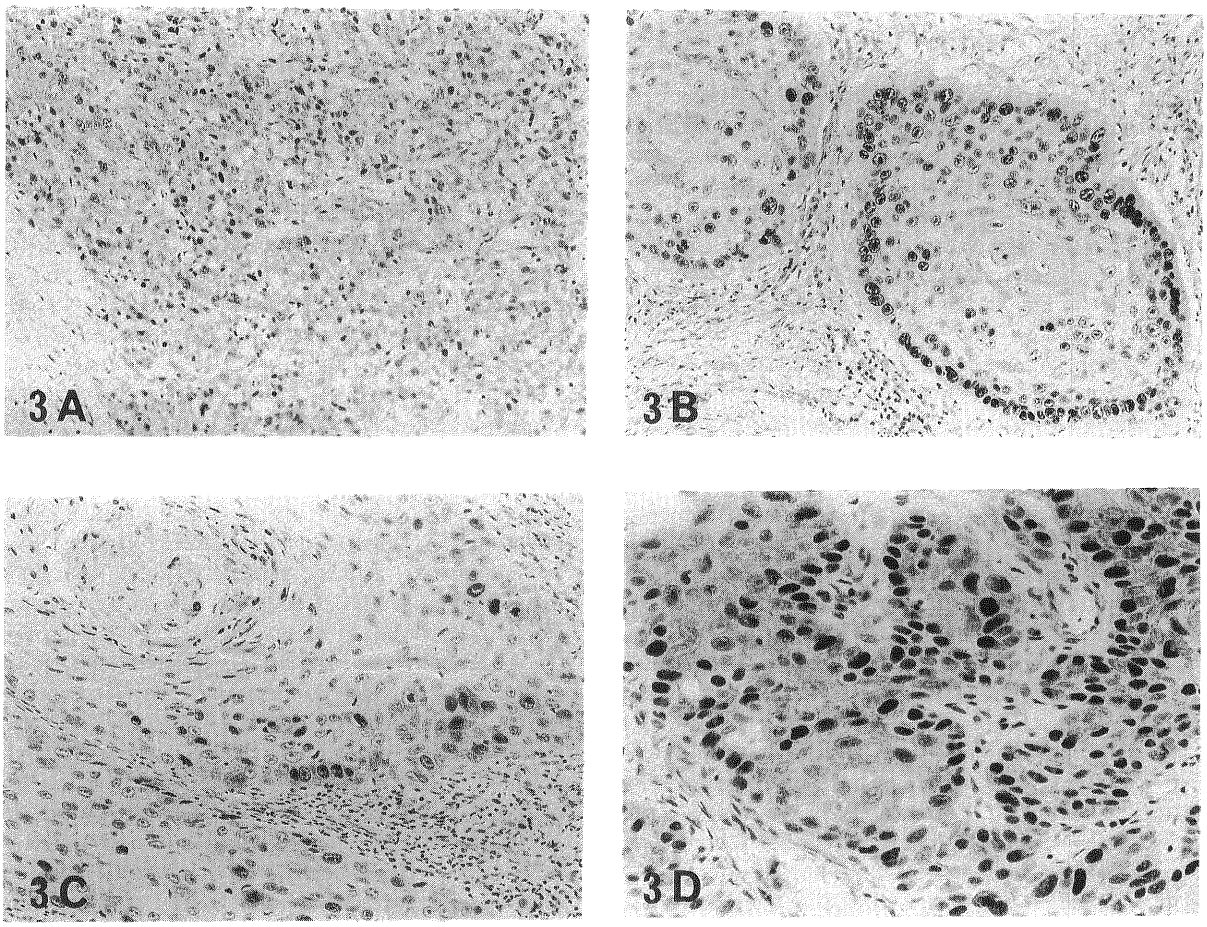

Fig. 3. Immunohistochemistry for p53. (A) Moderate to intense nuclear staining of the glandular epithelium in a feline mammary carcinoma (poorly differentiated type). × 150. (B) Moderate nuclear staining in a canine squamous cell carcinoma (common type). × 200. The reaction products are seen mainly in the peripheral neoplastic cells of tumor island. (C) Moderate nuclear staining in a canine squamous cell carcinoma (poorly differentiated type). × 200. (D) Intense nuclear staining in a feline squamous cell carcinoma. × 400.

lia, but there was no expression in the benign groups and normal mammary glands (Fig. 3A). Both canine and feline squamous cell carcinomas showed intense immunoreactivity with a frequency of about 40% in both cases (Figs. 3B, C and D). In the majority of positive cases in squamous cell carcinomas, the p53 nuclear staining was seen mainly in the peripheral cells of neoplastic foci (Fig. 3B). Poorly differentiated feline squamous cell carcinomas showed much higher frequency of p53 expression than the other lower grades, although there was no correlation between p53 expression and histological grade in canine squamous cell carcinomas. In contrast, p53 expression was not detected in any of the canine and feline basal cell tumors.

DISCUSSION

This study is the first demonstration of cyclin A overexpression in canine and feline tumors using immunohistochemical methods. Immunohistochemistry using cyclin A antibody made it possible to obtain detailed measurement of cyclin A expression rates and patterns in individual tumor cells, and provided a suitable method of screening for cyclin A abnormality.

The percentage expressing cyclin A among canine malignant mammary tumors (50%) and feline mammary carcinomas (48.6%) was very high. However, in human breast cancer, it is likely that the tumorigenesis correlate with the alterations of cyclin D1 and cyclin E rather than cyclin A, although there is a little report of aberrant cyclin A in human and murine mammary tumors [35, 36]. While canine squamous cell carcinomas (43.8%) and feline squamous cell carcinomas (90%) showed a high level of immunoreactivity for cyclin A. This finding was consistent with the results of previous investigations of cyclin A overexpression in human squamous cell carcinomas at different sites [9, 11, 20, 22, 44–46]. Except for 2 cases of Bowen's disease, all feline squamous cell carcinomas showed expression of cyclin A, this result perhaps depending on the difference in each carcinogenesis. Canine basal cell tumors usually have relative high mitotic activities and those of this study also did. However,

cyclin A expression was observed in only 17.6% canine basal cell tumors, and was undetectable in feline basal cell tumors.

How the unscheduled overexpression of cyclin A participates in tumor progression remains unknown. The presence of this anomalous condition in tumor cells may indicate either increased tumor proliferation (cyclin A being an integral component of the cell cycle), an alteration of its gene or protein upregulation. There are several reports on cyclin A as a marker of proliferative activity in cancer [9, 16]. Their reports concluded that the expression of cyclin A is a powerful prognostic factor in lung carcinoma [9] and soft tissue sarcomas [16].

The present immunohistochemical analyses for cyclin A suggested that cyclin A is frequently overexpressed in certain canine and feline tumors, such as mammary tumors and squamous cell carcinomas, and may have a role in the proliferation of their tumors. Furthermore, our findings indicated that cyclin A immunopositive reaction was more frequent in poorly differentiated tumors of feline mammary carcinomas and canine squamous cell carcinomas. This result may reflect that poorly differentiated tumors usually exhibit aggressive proliferation. The possibility is that the expression of cyclin A is related to their tumor progression, because strong and diffuse cyclin A immunoreactivity was relatively limited in malignant cases.

Derangement of the normal expression of cyclin D1 has been shown in a wide variety of human tumors, including breast cancers [1, 3, 8, 13, 21, 24, 30, 39, 48], head and neck cancers [4, 26] and esophageal cancers [18, 19, 32]. However, there was a distinct discrepancy in the numbers of cyclin D1 positive cases between our results and previous reports on human cancer. Of all the tumor samples investigated in the present study, the expression of cyclin D1 was detected in only 4 cases, including 2 canine mammary adenocarcinomas, one canine squamous cell carcinoma and one feline basal cell tumor, contrasting with the results of immunohistochemical analysis for cyclin A. Thus, no significant correlation was detected between cyclin D1 expression and some tumor types in the present study. We suggested that the altered expression of cyclin D1 occurs rarely in tumor types examined, although it is possible that in 4 positive cases the expression of cyclin D1 may play a potential role in the pathogenesis of their tumor.

In this study, as well as cyclin A, immunohistochemistry for p53 seemed to be a reliable method for evaluating p53 overexpression in canine and feline tumors. Under physiological condition, wild type p53 has a very short half-life measured in minutes. While, mutation at the p53 locus may lead to the synthesis of aberrant p53 protein (mutant form) with a prolonged half-life and increased stability. This accumulated protein is the target of immunological p53 detection [28, 31]. But it has been shown that also wild type p53 can have an elongated half-life time when coupled to another protein [33]. Because p53 antibody used recognize wild type and mutant p53, wild type p53 could theoretically constitute a part of the positively stained population.

We already have demonstrated that p53 immunoreactivity of canine mammary tumors occurred frequently in both benign mammary lesions (16%) and malignant tumors (30.6%), mainly adenocarcinomas [34]. In comparison, 7/37 (18.9%) feline mammary carcinomas showed p53 immunoreactivity, while none of the benign groups such as adenosis and fibroadenoma did so. About 40% of both canine and feline squamous cell carcinomas exhibited intense immunoreactivity for p53. There are other previous reports that expression of p53 protein showed in 29, 69% of canine squamous cell carcinomas [12, 40]. The percentage expressing p53 in our investigation was similar to that found by Teifke and Lohr [40], but much lower than that reported by Gamblin *et al.* [12], in spite of the fact that both studies used the same antibody.

In human tumors, positive immunohistochemical staining is often accepted as evidence of an underlying p53 genetic abnormality [2, 10, 37, 41]. Conversely, several reports have described p53 protein accumulation independently from genetic alterations within a wide range of human malignant tumors [6, 15]. In the present study, p53 expression appears to occur commonly in feline mammary carcinomas and squamous cell carcinomas of dogs and cats, and associate with their tumorigenesis. To more fully establish the role of p53 in the development of their tumors, further analyses of the genetic profiles of their tumors using molecular techniques is needed. Interestingly, the poorly differentiated types of feline mammary carcinoma and squamous cell carcinoma tended to associate with p53 expression compared to the other two types. Therefore, the possibility is that altered expression of p53 may correlate with differentiation of neoplastic cells of their tumors. However, because only a limited number of samples were examined, the significance of this observation requires further study.

A correlation between mutation of p53 and amplification of cyclin D1 has been demonstrated by many investigators in various human cancers, including breast cancer and squamous cell carcinoma [5, 14, 27, 47]. In addition, it has recently been reported that co-expression of cyclin A and p53 is associated with human endometrial carcinomas [38]. However, distinct co-expression among cyclin A, cyclin D1 and p53 was not detected in all the types of tumor examined.

Although molecular analysis for positive cases in this study remains to be done, this report demonstrates that immunohistochemistry for cyclin A and p53 is useful in detecting the expression in the canine and feline tumors, such as mammary tumors and squamous cell carcinoma. However, further investigations into cyclin A, cyclin D1 and p53 and other cell cycle-related oncogenes are needed to clarify their roles in the development of various tumors in dogs and cats.

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