NOTE *Pathology*

Amplification of the Cyclin A Gene in Canine and Feline Mammary Tumors

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ABSTRACT. DNAs from 33 canine mammary tumors and 8 feline mammary carcinomas were examined by Southern blot analysis to clarify genomic abnormalities of the cyclin A gene. Amplification of cyclin A was detected in 27.3% (9/33) of canine mammary tumors and 87.5% (7/8) of feline mammary carcinomas. It was suggested that amplification of cyclin A do not correlate directly with the tumorigenesis of canine mammary tumors, because there was no significant difference of incidence of cyclin A amplification between the benign and malignant tumors. In feline mammary carcinomas, the high frequency of cyclin A amplification raised the possibility that the amplification lead to the protein overexpression and play an important role in the tumorigenesis.

KEY WORDS: amplification, cyclin A, mammary tumor.

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Cyclins are prime cell cycle regulators that are central to the control of major checkpoints in eukaryotic cells. Cyclins are categorized into three types: A-type, B-type and G1 cyclins (C-, D-, and E-types), and are activated by forming a complex with cyclin dependent kinases (cdk) at various stages of the cell cycle. The involvement of several cyclins in human cancer has been recognized over the last several years [1–13, 15, 17–21].

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 [3]. Recently, cyclin A alterations have also been identified in several tumors including squamous cell carcinomas of the lung [6, 19–21], oral cavity [11], esophagus [7] and uterine cervix [17].

We recently found that overexpression of cyclin A protein occurs frequently in canine malignant mammary tumors and feline mammary carcinomas. However, molecular analysis of cyclin A remains to be done. Therefore, we examined DNAs from canine and feline mammary tumors for amplification of the cyclin A gene using Southern blot analysis.

The samples of 33 canine mammary tumors and 8 feline mammary carcinomas were collected during a 3-year period (1996–1998) at the Department of Veterinary Pathology, Miyazaki University, Japan. For histopathology, the samples were fixed in 10% formalin, and paraffin sections were prepared and stained with hematoxylin and eosin (HE). The histological typing of the tumors is listed in Tables 1 and 3.

Immunohistochemistry was performed by using Envision-Polymer reagent (Dako Japan, Kyoto, Japan). 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.). The chromogenic reaction was carried out with diaminobenzidine (Sigma, St. Louis, U.S.A.) and counterstained with Mayer's hematoxylin. Specimens with more than 10% of neoplastic cells showing positive immunoreactivity cells were considered positive.

We screened DNAs of 33 canine mammary tumors and 8 feline mammary carcinomas by Southern blotting for abnormalities of the cyclin A gene. The tissue samples were frozen in liquid nitrogen and stored at –80°C before being processed. Normal testes of 5 dogs and 5 cats were used as controls. High molecular weight DNA was isolated from frozen tissue specimens as described previously [16]. Briefly, tissue was homogenized in DNA extraction buffer, and digested with proteinase K and RNase. DNA was isolated after phenol/chloroform extraction and ethanol precipitation.

Ten μg of each DNA was digested with the restriction enzymes *Eco*RI, *Hind*III, *Bam*HI and *Pst*I, then electrophoresed through a 1% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham, UK), as described previously [16].

The human cyclin A probe (*Eco*RI-*Eco*RI fragment) was kindly provided by Dr. H. Matsushime. The DNA fragments were labeled with [α-^{32}P]dCTP using the random priming procedure (Takara, Kyoto, Japan) for use as a hybridization probe. The filters were hybridized to the probe at 37°C for 24 hr in hybridization solution [16], then incubated at 37°C in a final wash of 0.1 × SSC. Autoradiographs were exposed for 1–3 days at –80°C to Fuji RX-U X-ray film in a cassette with an intensifying screen.

To quantify the degree of amplification of cyclin A, 10 μg of digested tumor DNA was sequentially diluted and the intensity of the hybridized bands was compared with that obtained 10 μg of normal control. Tumor DNA samples showing a signal ratio of $\geqq 2\times$ that were observed in the normal control DNA were considered to be amplified.

Of 33 canine mammary tumors, cyclin A amplification was found in 9 cases (27.3%) comprising 4 benign mixed tumors (case Nos. 6, 7, 8 and 10), 3 adenocarcinomas (case Nos. 19,

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Table 1. Cyclin A amplification and immunohistochemical results of 33 canine mammary tumors

Case No.	Histopathological diagnosis	Amplification	Overexpression
1	Adenoma	–	–
2	Adenoma	–	–
3	Adenoma	–	ND
4	Adenoma	–	–
5	Adenoma	–	–
6	Benign mixed tumor	+	–
7	Benign mixed tumor	+	–
8	Benign mixed tumor	+	ND
9	Benign mixed tumor	–	ND
10	Benign mixed tumor	+	–
11	Benign mixed tumor	–	–
12	Benign mixed tumor	–	–
13	Benign mixed tumor	–	ND
14	Benign mixed tumor	–	–
15	Adenocarcinoma	–	–
16	Adenocarcinoma	–	+
17	Adenocarcinoma	–	+
18	Adenocarcinoma	–	–
19	Adenocarcinoma	+	–
20	Adenocarcinoma	–	ND
21	Adenocarcinoma	–	–
22	Adenocarcinoma	–	+
23	Adenocarcinoma	+	+
24	Adenocarcinoma	–	ND
25	Adenocarcinoma	–	ND
26	Adenocarcinoma	–	–
27	Adenocarcinoma	–	+
28	Adenocarcinoma	–	–
29	Adenocarcinoma	–	–
30	Adenocarcinoma	+	+
31	Malignant mixed tumor	–	–
32	Malignant mixed tumor	+	–
33	Malignant mixed tumor	+	+

* ND: not done.

23 and 30) and 2 malignant mixed tumors (case Nos. 32 and 33). The data on cyclin A amplification are summarized in Tables 1 and 2. Representative examples of cyclin A abnormalities in canine mammary tumors are shown in Fig. 1. *Eco*RI-digested DNA samples showed 3 band patterns (as shown in Table 2) in both normal and tumor samples, and revealed 2-fold amplification in case Nos. 6, 7, 8, 10 and 23. *Hind*III-digested DNA samples had 2 normal bands at 23.0 and 8.5 kb, and manifested 2- and 4-fold amplification in case Nos. 23 and 30, respectively. Digestion with *Bam*HI showed normal bands at 13.5, 5.6 and 2.7 kb, and 2 to 4-fold amplification was detected in case Nos. 6, 7, 19, 23, 30, 32 and 33. Digestion with *Pst*I resulted in normal bands at 15.0, 7.9, 5.7, 2.4 and 1.5 kb, and case Nos. 6, 7, 19, 32 and 33 had 2 to 6-fold amplified bands (Fig. 1). Digestion with *Bam*HI and *Pst*I also showed DNA polymorphism in only one adenocarcinoma (case No. 22) without amplification (Fig. 1).

On the other hand, all of the feline mammary carcinomas except for case No. 8 showed cyclin A amplification to a variable degree (4 to 8-fold). Thus, the incidence of cyclin A amplification was much higher than that in canine mammary tumors. The results of amplification are described in Tables 3 and 4. The amplification was clearly detected after digestion with any of the restriction enzymes used. Representative examples of cyclin A abnormalities are shown in Fig. 2. *Eco*RI-digested DNA samples showed normal bands at 15.0, 7.8 and 5.6 kb, *Hind*III-digested samples at 30.0, 11.0 and 8.5 kb, *Bam*HI-digested samples at 18.0 and 15.0 kb and *Pst*I-digested samples at 8.5, 4.4 and 2.4 kb (Fig. 2), respectively. Case Nos. 2, 4, 6 and 7 showing amplification also revealed an increased copy number of cyclin A at the same locus. While, in the case Nos. 1, 3 and 5, *Eco*RI-digested DNA samples showed extra bands at 15.0, 7.8, 5.6, 3.6 and 3.3 kb, *Hind*III-digested samples at 30.0, 11.0, 8.5 and 4.9 kb, *Bam*HI-digested samples at 18.0, 15.0 and 11.0 kb and *Pst*I-digested samples at 13.0, 8.5, 4.4 and 2.4 kb (Fig. 2), respectively.

In our study, cyclin A gene amplification was observed in about 27.3% of canine mammary tumors. However, there was no significant difference of incidence of cyclin A amplification between benign and malignant mammary tumors. In addition, the results of amplification were inconsistent with our recent immunohistochemical results (Table 1) indicating that cyclin A overexpression was present in 50% of malignant mammary tumors but absent in benign mammary tumors.

Table 2. Molecular weight of the detected bands in DNA obtained from canine mammary tumors using 4 types of restriction enzyme

Case / Enzyme	Normal band (kb)			No. 6	No. 7	No. 8	No. 10	No. 19	No. 22	No. 23	No. 30	No. 32	No. 33
*Eco*RI				amp: 2	amp: 2	amp: 2	amp: 2			amp: 2			
	6.5	6.5	6.5	6.5	6.5	6.5	6.5		6.5	6.5			
	6.4	6.4	6.4	6.4	6.4	6.4	6.4		6.4	6.4			
	5.6	5.6		5.6	5.6		5.6						
	4.5		4.5		4.5			4.5	4.5				
	3.4	3.4	3.4	3.4	3.4	3.4	3.4		3.4	3.4			
	1.1		1.1			1.1			1.1	1.1			
*Hind*III										amp: 2	amp: 4		
		23.0							23.0	23.0	23.0		
		8.5							8.5	8.5	8.5		
*Bam*HI				amp: 2	amp: 2			amp: 4		amp: 2	amp: 2	amp: 4	amp: 2
		13.5		13.5	13.5			13.5	9.2	13.5	13.5	13.5	13.5
		5.6		5.6	5.6			5.6	5.6	5.6	5.6	5.6	5.6
		2.7		2.7	2.7			2.7	4.2	2.7	2.7	2.7	2.7
									2.7				
									2.0				
Pst I				amp: 6	amp: 4			amp: 3				amp: 2	amp: 2
		15.0		15.0	15.0			15.0	18.0			15.0	15.0
		7.9		7.9	7.9			7.9	7.9			7.9	7.9
		5.7		5.7	5.7			5.7	5.7			5.7	5.7
		2.4		2.4	2.4			2.4	2.5			2.4	2.4
		1.5		1.5	1.5			1.5	2.4			1.5	1.5
									1.5				

Note: amp: number of amplified copies of cyclin A gene. The case number in this Table is correlated to Table 1 and Fig. 1.

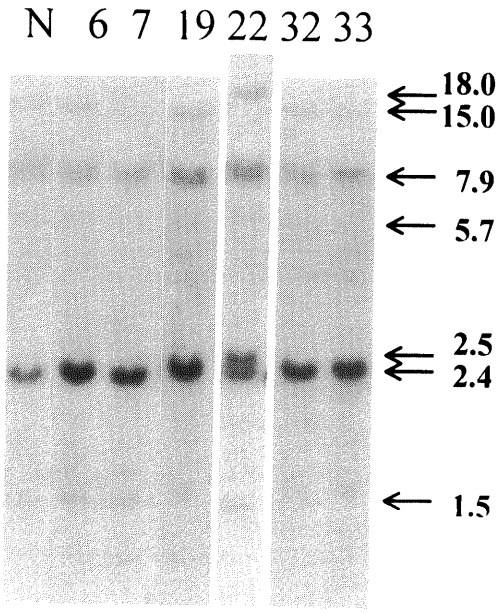

Fig. 1. Southern blot analysis of the genomic DNAs obtained from 6 canine mammary tumors (case Nos. 6, 7, 19, 23, 32 and 33) and normal canine testis (N) digested with *Pst*I, using the human cyclin A cDNA gene probe. Numbers on the right are the sizes of the hybridized bands; units are kilobase pair.

Unexpectedly, 4 of 7 canine malignant mammary tumors showing cyclin A overexpression had no detectable amplification. Such findings imply that mechanisms other than amplification of cyclin A may account for the increased expression of the protein. For example, expression of cyclin A may be reflected by mechanisms such as mutation in the promoter region or altered expression of a regulatory transcription factor. It was suggested that cyclin A amplification do not correlate with the protein overexpression in canine mammary tumors.

With one exception, all of the feline mammary carcinomas (87.5%) showed amplification of cyclin A. According to our immunohistochemical studies (Table 3), 3 of 5 cases showing amplification displayed expression of the gene products. However, neither of the remaining 2 cases showed overexpression of cyclin A in spite of the presence of amplification. Similar findings have been observed in some reports concerning amplification of cyclin D1 [1, 2, 13]. The most likely explanation is that some loss or disruption of the amplified allele presumably affected the expression of normal products of cyclin A gene. One case without amplification showed no staining for cyclin A. In feline mammary carcinomas, the high frequency of cyclin A amplification raised the possibility that the amplification leads to the protein overexpression and plays an important role in the tumorigenesis. Because of the small number of cases examined, the relationship of amplification to overexpression remains unclear. Therefore, further studies are needed to elucidate whether amplification of cyclin A correlates with its abundant expression in feline mammary carcinoma.

It is well known that amplification of cyclin D1 occurs commonly in human breast cancer [4, 5, 8, 10, 12, 18]. Therefore, we also studied the same tumor DNAs by Southern blot

Table 3. Cyclin A amplification and immunohistochemical results of 8 feline mammary carcinomas

Case No.	Histopathological diagnosis	Amplification	Overexpression
1	Carcinoma	+	+
2	Carcinoma	+	+
3	Carcinoma	+	+
4	Carcinoma	+	ND
5	Carcinoma	+	–
6	Carcinoma	+	–
7	Carcinoma	+	ND
8	Carcinoma	–	–

* ND: not done.

Table 4. Molecular weight of the detected bands in DNA obtained from feline mammary carcinomas using 4 types of restriction enzyme

Case Enzyme	Normal band (kb)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
*Eco*RI		amp: 2	amp: 4	amp: 2	amp: 2	amp: 2	amp: 2	amp: 2
	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6
		3.6		3.6		3.6		
		3.3		3.3		3.3		
*Hind*III		amp: 6	amp: 8	amp: 8	amp: 4	amp: 4	amp: 4	amp: 4
	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
		4.9		4.9		4.9		
*Bam*HI		amp: 6	amp: 6	amp: 6	amp: 4	amp: 4	amp: 5	amp: 5
	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
		11.0		11.0		11.0		
Pst I		amp: 6	amp: 6	amp: 6	amp: 6	amp: 2	amp: 4	amp: 2
		13.0		13.0		13.0		
	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4

Note: amp: number of amplified copies of cyclin A gene. The case number in this Table is correlated to Table 3 and Fig. 2.

analysis for amplification of the cyclin D1 gene. However, any of the canine and feline tumor samples in this study seemed to have no significantly increased cyclin D1 gene copy number (data not shown). The absence of detectable amplification of cyclin D1 was consistent with our immunohistochemical results indicating that cyclin D1 overexpression was very rare in both canine and feline tumors. In contrast to cyclin D1, there appear to have been no previous studies demonstrating amplification of cyclin A in human breast cancer. However, the results of that study deviated from ours, which demonstrated cyclin A amplification in 27.3% of canine mammary tumors and 87.5% of feline mammary carcinomas.

To our knowledge, there have also been no reports concerning amplification of cyclin A in canine and feline tumors. However, we have shown that amplification of cyclin A occurs frequently in canine and feline mammary tumors. We hope that further accumulation of knowledge will clarify the role of the cyclin A gene in the development of canine and feline tumors.

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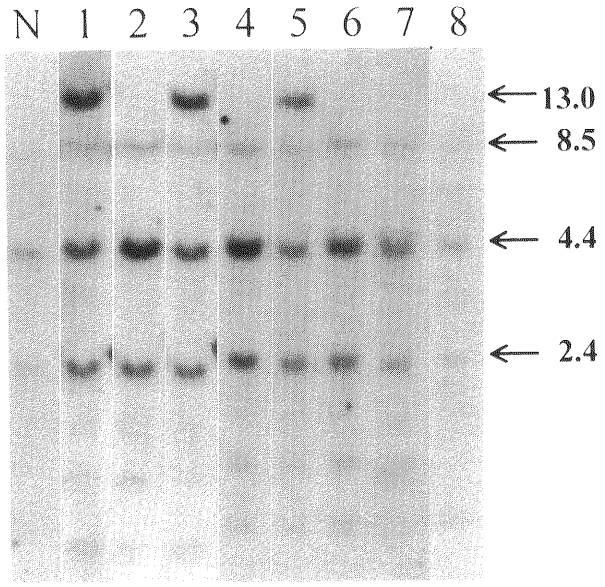

Fig. 2. Southern blot analysis of the genomic DNAs obtained from 8 feline mammary carcinomas (case Nos. 1–8) and normal feline testis (N) digested with *Pst*I, using the human cyclin A cDNA gene probe. Numbers on the right are the sizes of the hybridized bands; units are kilobase pair.

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