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Amplification of the c-*yes* Oncogene in Canine Mammary Tumors

Anudep RUNGSIPIPAT, Susumu TATEYAMA*, Ryoji YAMAGUCHI, Kazuyuki UCHIDA, Yuichi MURAKAMI, Noriaki MIYOSHI[1)] and Toshiharu HAYASHI[2)]

Departments of Veterinary Pathology, Faculty of Agriculture, Miyazaki University, Nishi 1–1, Gakuen Kibana Dai, Miyazaki, 889–2192, [1)]Kagoshima University, 1–21–24 Korimoto, Kagoshima, 890–0065, and [2)]Yamaguchi University, 1677–1 Yoshida, Yamaguchi, 753–8515, Japan

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ABSTRACT. Genomic DNAs of 14 mammary tumors were analyzed by Southern blot hybridization using a human c-*yes*-1 oncogene probe. The amplification was successful in half of the cases (7 adenocarcinomas). The degree of amplification was approximately 4-fold, and a high proportion was seen in malignant tumors. In addition, DNA polymorphism was detected in two adenocarcinomas.—KEY WORDS: c-*yes*, canine, mammary tumor.

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Mammary tumors are the most frequent neoplasms in female dogs, accounting for approximately 50% of all neoplasms [4, 10]. Several studies using immunohistochemistry, flow-cytometric DNA analysis and tissue culture have been conducted to clarify the mechanism of tumorigenesis [2, 7, 8]. Amplification or overexpression of several oncogenes such as c-*erb*B-2, c-*myc*, c-Ha-*ras*, N-*ras*, and c-*src* is reported to be associated with the development of human breast cancers [3, 5, 9, 15, 16]. In addition, overexpression of the c-*erb*B-2 oncogene is also reported to be correlated with the occurrence of malignant canine mammary tumors [1].

The human cellular *yes* oncogenes, c-*yes*-1, and c-*yes*-2 have been identified as homologues of v-*yes*, the oncogene of avian sarcoma virus Y73 [25]. The human c-*yes*-1 oncogene encodes a 62-kDa protein that is myristylated at its N-terminus, associated with membranes, and acts as a non-receptor protein tyrosine kinase [21, 22]. It has been reported that the c-*yes* oncogene is involved in human gastric cancer [19] and is actively transcribed in tumor cell lines. The notion that the c-*yes*-1 proto-oncogene is located in the chromosomes of clinically normal animals and amplified in canine mammary tumors and lymphoid leukemia is accepted [11–13, 28]. However, the relationship between the tumorigenesis and the c-*yes* oncogene in canine mammary tumors has not yet been confirmed and requires further investigation. Here we report an analysis of genomic DNA-level structural changes in the human c-*yes* oncogene and c-*yes* amplification in both benign and malignant mammary tumors in dogs.

Tissue samples: Canine mammary tumors removed surgically were collected during a 3-year period (1995–1997) at the Department of Veterinary Pathology, Miyazaki University. Tissue samples for oncogene analysis were stored at −70°C until DNA extraction. The tissue samples for histology were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections 4 μm thick were then stained with hematoxylin and eosin (HE). Histopathological diagnosis was based on the World Health Organization criteria [6]. Testes of clinically normal mongrel adult dogs and a human placenta were used as controls.

Immunohistochemistry: Immunohistochemistry was performed using a modified streptavidin biotin complex method (Histofine SAB kit[R], Nichirei, Japan). A rabbit polyclonal c-*yes* was used as the primary antibody. The sections of mammary tumors were immunostained, counterstained with Mayer's hematoxylin and observed under light microscope.

Isolation of DNA: High-molecular-weight genomic DNA was prepared according to the method described by Sambrook *et al.* [18]. Briefly, 1.0–1.5 g of each specimen was homogenized in DNA extraction buffer (10 mM Tris-hydroxymethyl amino methane-HCl (Tris-HCl), pH 8.0, 20 mM EDTA, 0.1 M NaCl and 0.5% sodium dodecyl sulfate (SDS), and digested with 20 mg/m*l* proteinase K (Wako, Japan) and 100 μg/m*l* RNase (Sigma, U.S.A.) for 16 hr at 37°C. The DNA was extracted 3 times with an equal volume of phenol/chloroform (1:1) and precipitated by addition of 2 volumes of 3 M Na acetate in absolute ethanol. Samples were resuspended in TE buffer pH 8.0 (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and analyzed for both concentration and purity spectrophotometrically.

Gel electrophoresis and Southern transfer: High-molecular-weight genomic DNA (10 μg) was digested with *Eco*RI, *Hind*III, *Bam*HI and *Pst*I restriction endonuclease enzymes according to the recommendation of the supplier (Wako, Japan), and subjected to electrophoresis on 1.0% agarose gel. Phage λ DNA digested with *Hind*III (Wako, Japan) was used as a molecular weight marker. The DNA samples were denatured with an alkaline reagent (1.5 M NaCl and 0.5 M NaOH) and transferred to a nylon membrane (Hybond N+, Amersham, UK).

DNA probe: The human c-*yes*-1 probe was a 0.54-kilobase (kb) *Hind*III-*Eco*RI cDNA fragment and showed 92% homology with the nucleotide sequence of the canine c-*yes* gene (provided by Dr. M. Shibuya, Department of Genetics, Institute of Medical Science, University of Tokyo).

* CORRESPONDENCE TO: TATEYAMA, S., Department of Veterinary Pathology, Faculty of Agriculture, Miyazaki University, Miyazaki, 889–2192, Japan.

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Human c-*yes*-1 cDNA was transformed into a competent bacterial host, *Escherichia coli* strain HB101 using pUC 119 as a vector. The DNA probe was labeled with α-^{32}P deoxycytidine-5'-triphosphate (approximate 111 TBq/mmol, ICN Biomedical Inc., California, U.S.A.) by the primer extension method with a random primer (Takara, Kyoto, Japan), and subjected to Sephadex-G-50 column chromatography (Pharmacia, Uppsala, Sweden). Specific activity was more than 1×10^9 CPM/μg template DNA.

Hybridization condition: The filters were prehybridized in a prehybridization solution (30% formamide, 1 M NaCl, 1% SDS, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 × Denhardt' s solution (0.2% each of bovine serum albumin (Sigma, U.S.A.), ficoll (Sigma, U.S.A.) and polyvinylpyrolidone (Sigma, U.S.A.)) and 250 μg/m*l* denatured salmon DNA (Sigma, U.S.A.) at 37°C for 16 hr. Hybridization was performed under low-stringency conditions in hybridization solution (prehybridization solution plus 5% dextran sulfate and ^{32}P labeled probe) at 37°C for 24 hr. The filters were washed successively; once with solution I (2x SSC, 1% SDS and 1x Denhardt's solution) at 37°C for 1 hr, twice with solution II (0.1x SSC and 1% SDS) at 37°C for 30 min and a final rinse in 0.1x SSC at room temperature for 5 min. The filters were dried at room temperature and then autoradiographed at −70°C for varying lengths of time using a Fuji intensifying screen and Fuji RX-U X-ray film (Fuji, Japan). To quantify the degree of amplification of c-*yes*-1, 10 μg of digested tumor DNA was sequentially diluted and the intensity of the hybridized bands was compared with that obtained with 10 μg of normal canine control.

Fourteen singly digested tumor DNA specimens were subjected to Southern blot hybridization, using *Eco*RI, *Pst*I, *Hind*III and *Bam*HI restriction enzymes. The unique hybridized bands were detected in 5 benign (4 benign mixed tumors and 1 complex adenoma) and 9 malignant tumors (8 adenocarcinomas and 1 malignant mixed tumor), and amplification was found in 7 malignant tumors. All of these cases did not show evidence of metastasis. The results are shown in Table 1. *Eco*RI-digested DNA samples showed unique bands at approximately 8.0, 3.2 and 3.0 kb compared with canine and human controls. A band about 4 times as intense at 3.2 kb was detected in case Nos. 9 and 12 (Fig. 1, lanes 9 and 12). *Hind*III-digested DNA samples had 2 normal bands at 5.0 and 3.7 kb and abnormal or amplified bands in each sample. Case Nos. 6 and 8 (Fig. 2, lanes 6 and 8) had about a 4-fold amplified band at 5.0 kb. Case No. 12 (Fig. 2, lane 12) had about a 4-fold amplified band at 3.7 kb. The cases showing DNA polymorphism were case No. 9 (Fig. 2, lane 9) with 4-fold overexpressed bands at 8.7, 6.2 and 3.7 kb, and case No. 13 (Fig. 2, lane 13) with 8-fold overexpressed bands at 6.2, 5.0 and 3.7 kb. Digestion with *Bam*HI resulted in normal bands at 15.0 and 4.0 kb, and 7 adenocarcinomas (Fig. 3, lanes 6, 8, 9, 10, 11, 12, and 13, respectively) had about a 4-fold amplified band at 4.0 kb. Otherwise, case No. 8 also had abnormally overexpressed bands at 3.6 and 0.5 kb. Digestion with *Pst*I enzymes resulted in normal bands at 4.2, 3.8 and 2.2 kb. Case Nos. 6 and 12 (Fig. 4, lanes 6 and 12) had about a 2-fold amplified band at 3.8 kb. The results are summarized in Table 2.

About 50% of all selected cases (7 adenocarcinomas) showed amplification of the c-*yes*-1 oncogene, and DNA polymorphism was detected in 2 adenocarcinomas, similar to observations reported for 6 solid canine mammary carcinomas [13]. The c-*yes* oncogene is known to be well expressed in all human tissues and organs, suggesting that the c-*yes* gene also has a role in the proliferation and/or differentiation of animal cells [21, 23, 26]. Thus, the amplified c-*yes*-1 DNA in cancer cells may result in elevation of c-*yes*-1 mRNA and might disturb the normal regulation of cell proliferation [19]. The degree of amplification of c-*yes*-1 was relatively low in these canine mammary tumors. However, it is noteworthy that the elevated level of c-*yes*-1 DNA was due to extreme polyploidy of chromosome 18 in these cancer cells, where c-*yes*-1 is reported to be located [19, 22]. The nucleotide sequence of the canine c-*yes* oncogene showed 92% and

Table 1. c-*yes* amplification and immunohistochemical results of 14 canine mammary tumors

Case No.	Age (yrs)	Histopathological diagnosis	Amplified cases[a)]	Immunohistochemical result[b)]
1	11	Benign mixed tumor	−	−
2	10	Benign mixed tumor	−	−
3	10	Benign mixed tumor	−	+
4	15	Benign mixed tumor	−	+
5	5	Complex adenoma	−	+
6	17	Adenocarcinoma	+	+
7	10	Adenocarcinoma	−	−
8	11	Adenocarcinoma	+	−
9	9	Adenocarcinoma	+	+
10	10	Adenocarcinoma	+	−
11	12	Adenocarcinoma	+	−
12	21	Adenocarcinoma	+	−
13	8	Adenocarcinoma	+	−
14	11	Malignant mixed tumor	−	−

a) −; normal hybridization, +: amplification, b): −; negative, +; positive.

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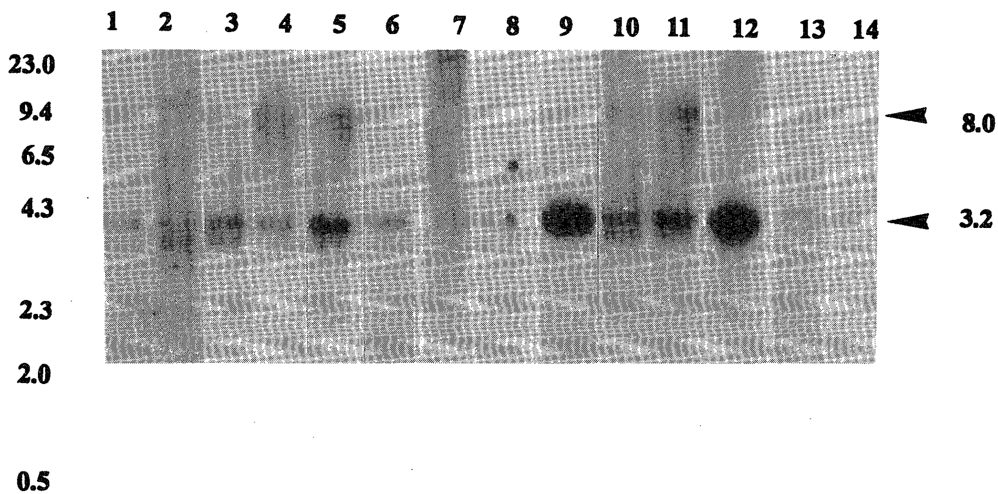

Fig. 1. Southern blot analysis of the genomic DNAs obtained from 14 canine mammary tumors digested with *Eco*RI, using the human c-*yes*-1 cDNA oncogene probe. Numbers on the right are the sizes of the hybridized bands and the numbers on the left are the sizes of the λ-DNA fragments digested with *Hind*III for use as size markers; units are kilobase pair.

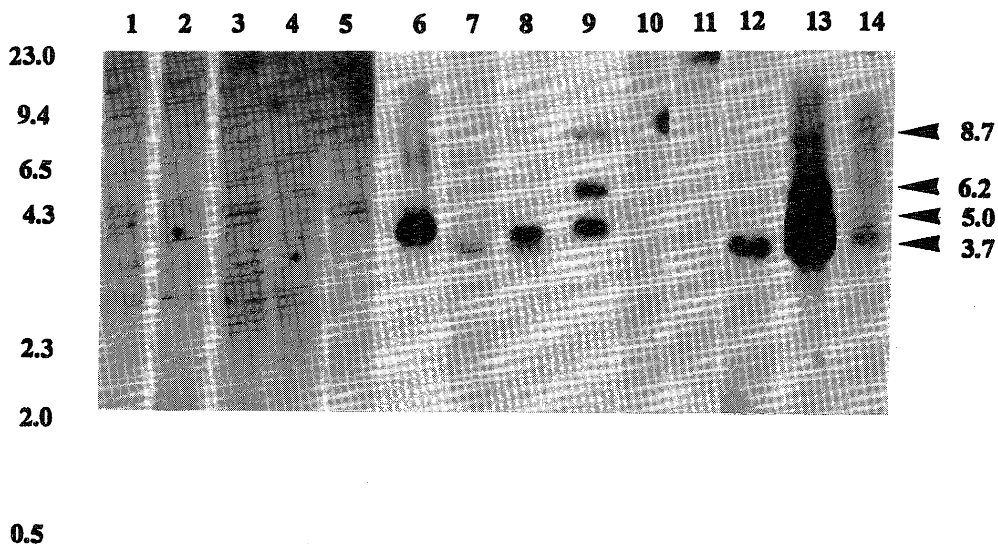

Fig. 2. Southern blot analysis of the genomic DNAs obtained from 14 canine mammary tumors digested with *Hind*III, using the human c-*yes*-1 cDNA oncogene probe. Numbers on the right are the sizes of the hybridized bands and the numbers on the left are the sizes of the λ-DNA fragments digested with *Hind*III for use as size markers; units are kilobase pair.

89.1% homology with those of the human c-*yes*-1 and chick c-*yes* genes previously reported [27]. Therefore, amplification of the human c-*yes*-1 oncogene in canine mammary tumors leads to the suggestion that the increase of the c-*yes*-1 oncogene may be involved in the transformation and maintenance of the transformed state of mammary epithelial cells. In our study, the main unique bands closely resembled the abnormal bands reported in canine mammary tumor and lymphoid leukemia [11, 13], although some differences were observed. This seemed to be attributable to variation in migration of the λ-DNA fragments used as molecular weight markers during electrophoresis [12, 28].

Because the amplified bands did not show the same pattern in each of the 7 tumors, amplification of the canine c-*yes* genomic DNA may consist of partial amplification or involve some form of rearrangement. In our previous immunohistochemical study (Table 1), we observed that only 2 of 7 cases showing genomic DNA amplification of the c-*yes* oncogene demonstrated expression of the oncogene product. Our results suggest that c-*yes*-1 oncogene amplification at the DNA level is reliably associated with

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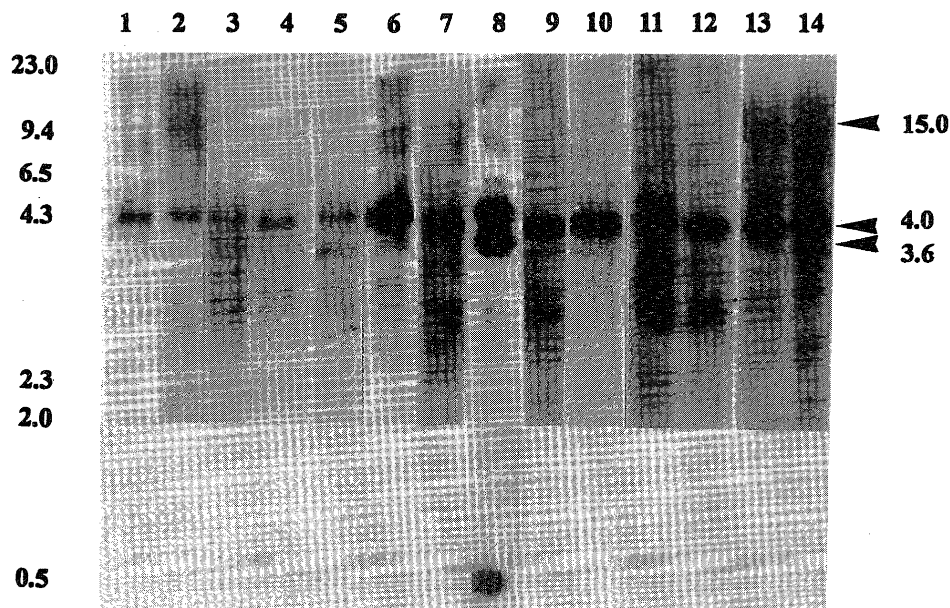

Fig. 3. Southern blot analysis of the genomic DNAs obtained from 14 canine mammary tumors digested with *Bam*HI, using the human c-*yes*-1 cDNA oncogene probe. Numbers on the right are the sizes of the hybridized bands and the numbers on the left are the sizes of the λ-DNA fragments digested with *Hind*III for use as size markers; units are kilobase pair.

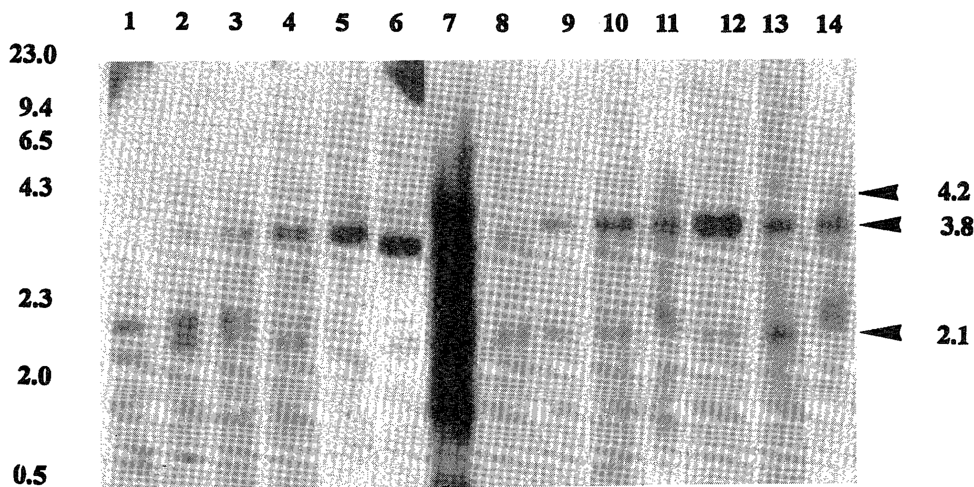

Fig. 4. Southern blot analysis of the genomic DNAs obtained from 14 canine mammary tumors digested with *Pst*I, using the human c-*yes*-1 cDNA oncogene probe. Numbers on the right are the sizes of the hybridized bands and the numbers on the left are the sizes of the λ-DNA fragments digested with *Hind*III for use as size markers; units are kilobase pair.

overexpression. We also observed that 5 cases showed amplification without overexpression, whereas 3 cases demonstrated c-*yes* overexpression, in the absence of amplification. Two possible reasons for these observations can be suggested. First, if a gene is only partially amplified, the tumor cells cannot express the normal product of the c-*yes* oncogene or may express an abnormal product. Second, other defects often occur in tumors, such as transcription errors or mutations in the regulation or promoter sequences, which could result in increased expression of the protein without amplification of the gene [17, 20, 24].

These findings may partly account for the inconsistency observed in the frequency of amplification and overexpression of the c-*yes*-1 oncogene. The notion that activation of c-*yes* tyrosine kinase plays an important role in mammary tumorigenesis is supported by a number of observations [14, 15] and we were able to detect the abnormal amplified structure in canine mammary tumors.

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Table 2. Molecular weight (kb) of the detected bands in DNA obtained from canine mammary tumors using 4 types of restriction endonuclease enzyme in this study

Case Enzyme	Normal band (kb)	No. 6	No. 8	No. 9	No. 10	No. 11	No. 12	No. 13
*Eco*RI	8.0							
	3.2			3.2*			3.2*	
	3.0			amp: 4			amp: 4	
*Hind*III	8.7			8.7				6.2
	5.0	5.0*	5.0*	6.2*				5.0*
	3.7	amp: 4	amp: 2	3.7*			3.7*	3.7*
			amp: 4			amp: 4	amp: 8	
*Bam*HI	15.0							
	4.0	4.0*	4.0*	4.0*	4.0*	5.0*	4.0*	4.0*
		amp: 4	3.6*	amp: 4	amp: 4	4.0*	amp: 4	amp: 4
			amp: 4			amp: 4		
*Pst*I	4.2							
	3.8	3.8*					3.8*	
	2.2	amp: 2					amp: 2	

Note: *: the amplified band, amp: number of amplified copies of c-*yes*-1 oncogene. The case number in this Table is correlated to Table 1 and Figs. 1–4.

To clarify the exact role of the c-*yes* oncogene in canine mammary tumor, further investigation using Northern blot hybridization and *in situ* hybridization are on progress.

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