Immunohistochemical Analysis of c-*yes* and c-*erb*B-2 Oncogene Products and p53 Tumor Suppressor Protein in Canine Mammary Tumors

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ABSTRACT. In order to evaluate the involvement of c-*yes* and c-*erb*B-2 oncogene products, and p53 tumor suppressor protein in canine mammary neoplastic lesions, sections of archived paraffin-embedded samples of 79 mammary tumors were analyzed immunohistochemically using antibodies against human c-*yes* p62 and c-*erb*B-2 products and p53. These 79 tumors were divided into 2 groups: 32 benign (2 adenosis, 7 simple adenomas, 14 complex adenomas, and 9 benign mixed mammary tumors) and 47 malignant tumors (26 simple adenocarcinomas, 7 complex adenocarcinomas, 5 solid carcinomas, 2 sclerosing carcinomas, 6 malignant mixed mammary tumors, and 1 malignant myoepithelioma). As a result of immunostaining, 40.6% (13/32) of the benign tumors and 21.3% (10/47) of the malignant tumors expressed the c-Yes oncogene product, ErbB-2 expression was detected in 50% (16/32) of the benign tumors and in 19.1% (9/47) of the malignant tumors. P53 expression was detected in 16% (4/25) of the benign tumors and in 30.6% (11/36) of the malignant tumors. Co-expression of c-Yes and ErbB-2, ErbB-2 and p53, and all 3 products was detected in 6, 1 and 7 tumors, respectively.—KEY WORDS: canine, mammary tumor, oncogene.

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Mammary tumors are the most common neoplasm in female dogs, accounting for approximately 50% of all neoplasms [8, 18]. Recently, there has been considerable interest in the role of oncogenes and tumor suppressor genes in the etiology and progression of many carcinomas, as studies on these genes may provide useful information on the course of malignant diseases and help with treatment selection [13, 19]. Changes in the structure and expression of proto-oncogenes have been shown to be involved in both the onset and progression of tumors [30]. One such mechanism is the amplification and overexpression of members of the protein kinase family of genes. These genes control cell growth by phosphorylating tyrosine or serine/threonine residues on proteins and represent many of the known cellular oncogenes and growth factor receptors. Several tyrosine kinase genes, including c-*yes* and c-*erb*B-2 have been found to be amplified in human breast cancers [6, 15, 28, 33].

The p53 tumor suppressor gene, located on human chromosome 17p13.1, is the single most common target for genetic alteration in human cancers [7, 22] and mutation of this gene is one of the most frequent genetic aberrations in human epithelial tumor systems, including breast carcinomas [9, 35, 36]. Furthermore, altered p53 expression in breast carcinomas was found to be associated with high-grade and estrogen receptors negative tumors [3] and evidence was indicating that the status of p53 is an important significant prognostic factor when p53 protein is accumulating in breast carcinomas [2, 10, 31, 34].

In previous studies, attempts have been made to use immunohistochemical techniques to detect ErbB-2 and p53 proteins in routinely processed mammary cancer specimens [9, 32]. DNA hybridization and dot blot analysis of mRNA have been carried out to determine the presence of gene amplification in canine mammary tumors cell lines and tumor tissues [1]. However, easier and more reliable techniques are needed for clinical purposes. To date, there has been no well-established immunohistochemical technique for the analysis of c-Yes, ErbB-2 and p53 in canine mammary tumors. The aim of this study was to examine and evaluate the involvement of c-Yes and ErbB-2 oncogene products and p53 tumor suppressor protein in canine spontaneous mammary tumors.

MATERIALS AND METHODS

Tissue: Tissue biopsy specimens of 79 canine mammary tumors taken during the period 1994-1997 were obtained from the Department of Veterinary Pathology, Faculty of Agriculture, Miyazaki University, Japan. Clinical history data, including the dogs' ages, tumor locations, sizes and the clinical stages accessible according to the staging system of Rosen and Oberman [26] were recorded. Slides were produced from archived paraffin-embedded tissue blocks and stained with hematoxylin and eosin in a routine manner. Histopathologic examination, diagnosis and tumor classification were carried out according to the World Health Organization criteria for histologic typing of canine mammary tumors [12].

Immunohistochemistry: Immunohistochemistry was performed using a modified streptavidin-biotin complex method (SAB) (Histofine SAB kit^{R}, Nichirei, Tokyo, Japan).

A rabbit polyclonal c-Yes antibody raised against a peptide corresponding to the amino acid terminal domain of c-Yes p62 of human origin (Santa Cruz Biotech, U.S.A.), a rabbit anti-human polyclonal antibody against the intracytoplasmic domain of ErbB-2 oncoprotein (DAKO, Denmark) and the CM1 polyclonal rabbit anti-human p53 antibody against wild and mutant forms of p53 (Novocastra, UK) were used as the primary antibodies.

Hydrated autoclave treatment was performed to enhance the immunoreactivities of ErbB-2 and p53. The sections used for c-Yes immunostaining were predigested with 0.1% (w/v) trypsin (Difco, U.S.A.) in Tris- hydrochloride buffer pH 7.6 containing 0.1% (w/v) $CaCl_2$ for 30 min at 37°C. Endogenous peroxidase activity was reduced by immersing the sections in a solution of 3% (v/v) hydrogen peroxide in methanol. The sections were incubated with normal goat serum for 30 min at 37°C, the primary antibody overnight at 4°C, the biotinylated secondary antibody for 30 min at 37°C and then with the SAB solution for 30 min at 37°C. Finally, the sections were exposed to the chromagen; 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.

The grading scale for interpretation of c-Yes and ErbB-2 was defined as follows: +1 weak membrane staining; +2, moderate membrane and weak cytoplasmic staining; +3, intense membrane and moderate to intense cytoplasmic staining and +4, intense membrane and cytoplasmic staining. Specimens with fewer than 20% of their cells showing positive immunoreactivity cells were considered negative [17, 22].

The intensity of p53 overexpression was assessed using a grading system based on the percentage of p53 positive nuclei. Tumors were assigned a score of 0 to 3: 0, tumor with no nuclear staining; 1, tumor with <10% of its nuclei stained; 2, tumor with 10 to 50% of its nuclei stained; 3, tumor with >50% of its nuclei stained. Tumors assigned scores of 2 to 3 were considered positive [9, 27].

RESULTS

Seventy-nine canine mammary tumors were divided into 2 groups comprising 32 benign mammary lesions and 47 malignant tumors. The mean ages of 70 dogs were 10.13 (range, 2 to 21) years. The mean ages at diagnosis of the benign and malignant groups were 9.18 (range, 4 to 13) years and 10.80 (range, 2 to 21) years, respectively. There was no significant difference between the tumor locations of the two groups. About 20% of the malignant group had metastasized into the adjacent lymph nodes. The clinical data are summarized in Table 1.

The histological classifications of the 32 benign mammary tumors were 7 simple adenomas, 14 complex adenomas, 2 adenosis and 9 benign mixed tumors. The 47 malignant tumors were categorized as 40 adenocarcinomas which were subdivided into 26 simple papillary types, 7 complex types, 5 solid carcinomas and 2 sclerosing carcinomas, 6 malignant mixed tumors and 1 malignant myoepithelioma. The histological data are presented in Table 2.

Table 1. Clinical features of 79 canine mammary tumors

	Benign tumor (total 32 cases)	Malignant tumor (total 47 cases)
Age (years)	4–13 (mean=9.18)	2–21 (mean=10.8)
2–21 (mean=10.13)		
<10 years old	43.8% (14/32)	29.8% (14/47)
≥10 years old	43.8% (14/32)	59.6% (28/47)
no data	12.4% (4/32)	10.6% (5/47)
Tumor locations		
Focal ipsilateral	40.6% (13/32)	36.2% (17/47)
Diffuse bilateral	43.8% (14/32)	38.3% (18/47)
no data	15.6% (5/32)	25.5% (12/47)

Table 2. Histological typing of 79 canine mammary tumors

Mammary tumor	Number of cases
Benign tumor	32 (40.5%)
Adenoma, simple	7 (8.9%)
Adenoma, complex	14 (17.7%)
Benign mixed tumor	9 (11.4%)
Adenosis with inflammation	2 (2.5%)
Malignant tumor	47 (59.5%)
Adenocarcinoma, simple	26 (32.9%)
Adenocarcinoma, complex	7 (8.9%)
Solid carcinoma	5 (6.3%)
Sclerosing carcinoma	2 (2.5%)
Malignant mixed tumor	6 (7.6%)
Malignant myoepithelioma	1 (1.3%)
Total	79

The immunohistochemical results are summarized in Table 3. Expression of the c-Yes was detected in the glandular epithelium of 29.1% (23/79) of the mammary tumors. Immunoreactivity was invariably seen in the cytoplasm and its intensity was variable. Thirteen cases (6 simple adenomas, 6 complex adenomas and 1 benign mixed tumor) of the 32 (40.6%) benign tumors were c-Yes positive (Fig. 1). Expression of c-Yes was observed in 21.3% (10/47) of the malignant tumors with 8 adenocarcinomas (Fig. 2) and 2 malignant mixed tumors almost fulfilling the criterion for c-Yes positivity.

Positive ErbB-2 oncogene immunoreactivity was observed in 31.6% (25/79) of the mammary tumors examined. This cell membrane staining pattern was observed exclusively in neoplastic cells, and positive staining in most cases was distributed uniformly throughout the neoplastic cell population, although the immunostaining intensities of cells of individual tumors showed variations. Fifty percent (16/32) (7 simple adenomas, 7 complex adenomas and 2 benign mixed tumors) of the benign group showed positive immunostaining (Fig. 3), whereas only 19.1% (9/47) of the malignant tumors, 8 of which were

Table 3. Expression of c-*yes* and c-*erb*B-2 oncogene products and p53 tumor suppressor protein detected by immunohistochemical analysis

Type of mammary tumor	Percentage of positive cases (numbers)		
	c-*yes*	c-*erb*B-2	p53
Benign tumor	40.6% (13/32)	50% (16/32)	16% (4/25)
Adenoma, simple	7.6% (6)	8.9% (7)	3.3% (2)
Adenoma, complex	7.6% (6)	8.9% (7)	1.6% (1)
Benign mixed tumor	1.3% (1)	2.5% (2)	1.6% (1)
Adenosis with inflammation	–	–	–
Malignant tumor	21.3% (10/47)	19.1% (9/47)	30.6% (11/36)
Adenocarcinoma, simple	5.1% (4)	7.6% (6)	8.2% (5)
Adenocarcinoma, complex	3.8% (3)	2.5% (2)	3.3% (2)
Solid carcinoma	–	–	3.3% (2)
Sclerosing carcinoma	1.3% (1)	1.3% (1)	1.6% (1)
Malignant mixed tumor	2.5% (2)	–	1.6% (1)
Malignant myoepithelioma	–	–	–
Total	29.1% (23/79)	31.6% (25/79)	24.6% (15/61)

adenocarcinomas (Fig. 4), were positive.

Expression of p53 protein was found 24.6% (15 of the 61 mammary tumors examined). The distribution of p53-immunoreative nuclei was usually uniform and cytoplasmic staining was seen in some specimens. Four (2 simple adenomas, 1 complex adenoma and 1 benign mixed tumor) of 25 (16%) of benign tumors showed positive p53 immunostaining and 11 (10 adenocarcinomas and 1 malignant mixed tumor) of 36 (30.6%) of malignant tumors expressed p53 (Figs. 5 and 6). Cytoplasmic staining was observed in 5 adenomas and 5 adenocarcinomas. The malignant myoepithelioma was not immunostained by either of the 3 antibodies.

Positive reactions for c-Yes, ErbB-2 or p53 alone were seen in 11, 10, and 5 tumors respectively. Co-expression of c-Yes and ErbB-2 was detected in 6 tumors, only one co-expressed ErbB-2 and p53, and 7 co-expressed c-Yes, ErbB-2 and p53.

DISCUSSION

The incidence of canine mammary tumors generally increases with age and such tumors occur rarely in dogs less than 2 years old [20]. The average age was 10.13 years ranging from 2 to 21 years at the time of tumor excision, in the dogs we studied, and the findings were comparable to those reported previously [5]. The present immunohistochemical analysis by using specific antibodies was accepted and had specificity to the canine mammary tumor examined here. Although the lack of sensitivity occurred through high background staining or no cell membrane immunoreactivity, this could be due to differences in fixation, the time lapse from excision to fixation, the actual time that the tissue is in a fixative or in the immunohistochemical detection system [17, 32]. Expression of the c-Yes was detected in 40.6% and 21.3% of both benign and malignant tumors, respectively. The c-Yes has been found to be expressed in various epithelial cells of normal tissues being believed to play a role in the transmission of signals originating from activated cell surface receptors that play important roles in proliferation and differentiation of cells [25, 38]. There may be a causal relationship between c-*yes* oncogene activity and canine mammary carcinogenesis, but the role of the c-*yes* oncogene in the pathogenesis of mammary tumors remains to be elucidated.

Analysis of the 79 canine mammary tumors revealed ErbB-2 expression in about 50% of the benign tumors, such as adenomas of the simple and complex types and 19.1% in the adenocarcinomas. The percentage of canine malignant tumors expressing ErbB-2 in this study was of the same order as that of human mammary cancers (20% to 30%), but lower than that of canine mammary tumors (74%) reported previously [14, 17, 21–24]. Nakopoulou *et al.* [22] demonstrated that ErbB-2 membrane staining of breast cancers was due generally to oncogene amplification, although gene amplification does not seem to be the only underlying genetic alteration responsible for ErbB-2 overexpression. In the majority of breast carcinomas, both gene amplification and overexpression of the protein product from single copy genes are events maintained during subsequent steps of tumor progression [4, 16, 29]. These findings suggest that ErbB-2 expression may be activated during the initial step of canine mammary tumor development, playing a role in malignant tumor development and that the ErbB-2 expression status may be a useful prognostic indicator in dogs with mammary tumors [1].

In humans, a direct correlation between p53 tumor suppressor gene overexpression and the histological grade of breast cancer has been demonstrated repeatedly. In our study, p53 immunoreactivity occurred frequently in both benign mammary lesions (16%) and malignant tumors (30.6%), mainly in adenocarcinomas. These results are similar to data derived from similar immunohistochemical

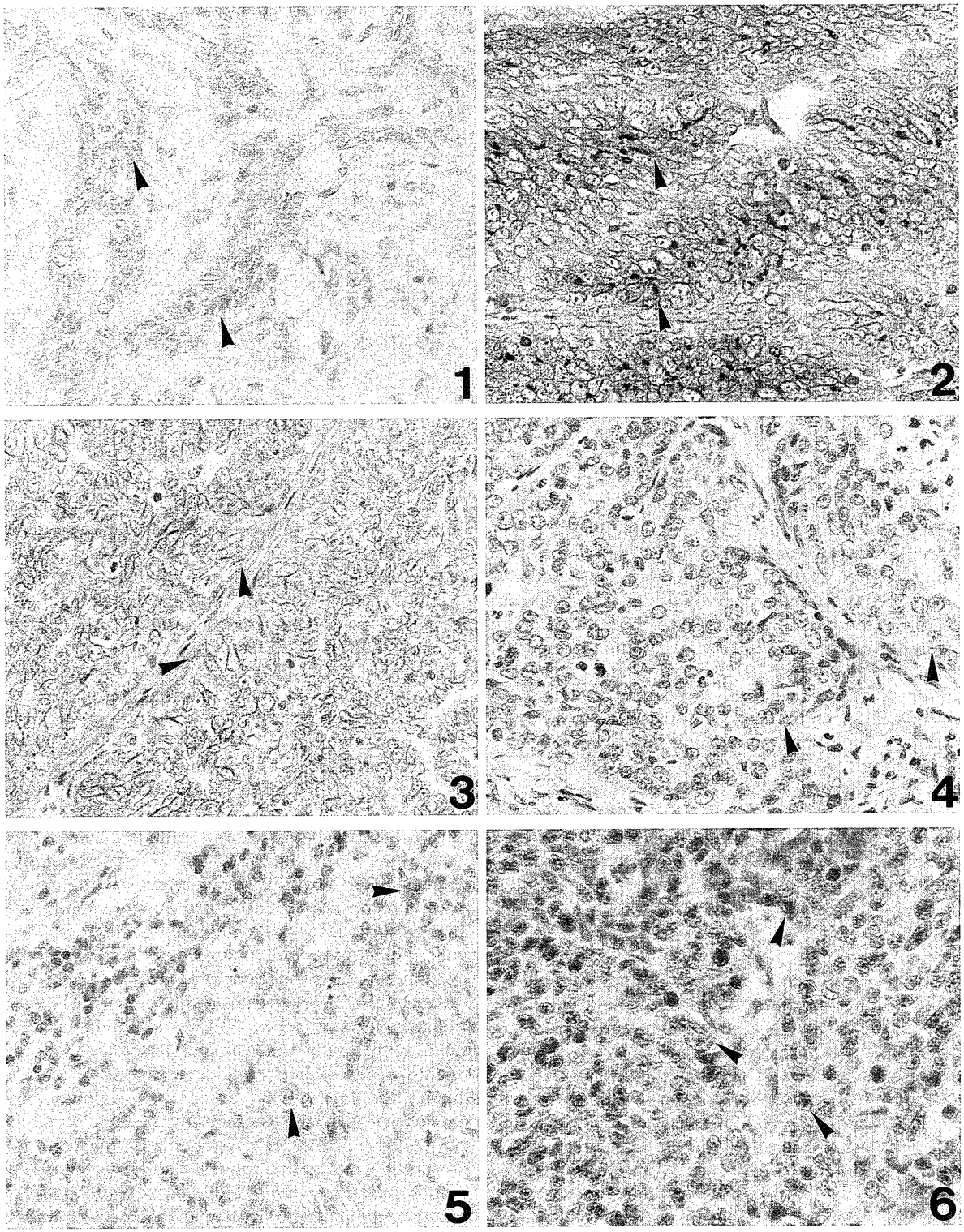

Fig. 1. Immunohistochemistry of a complex adenoma with c-Yes expression, arrowheads indicate positive cytoplasmic staining. (SAB immunoperoxidase method, counterstained with Mayer's hematoxylin, × 400)

Fig. 2. Tumor cells of an adenocarcinoma show positive immunoreactivity with c-Yes (arrowheads). (SAB immunoperoxidase method, counterstained with Mayer's hematoxylin, × 400)

analyses of human breast cancers that showed the incidence of the p53 positive staining of carcinomas was significantly higher than that of adenomas [3, 22, 34]. The different values for canine and human tumors may be attributable to the different antibodies and procedures used. Possible reasons for the high proportion of p53-expressing canine benign mammary tumors may include inappropriate detection of non-p53 protein and/or detection of the non-tumorigenic wild-type p53 protein [27, 37]. Our study showed a distinct association of p53 overexpression with lymph node metastasis in a complex adenocarcinoma and a solid adenocarcinoma. Thus, overexpression of p53 occurs at all stages of breast cancer and as p53 is directly involved in important cellular functions, including regulation of the cell cycle, alteration of the p53 gene may be an important step in the initiation of canine mammary tumors.

We observed co-expression of the c-*yes* and c-*erb*B-2 oncogenes and of c-*yes*, c-*erb*B-2 and p53 in 6 and 7 canine mammary tumors, respectively. It is conceivable that co-expression of c-Yes and ErbB-2 in these 13 tumors obviated the requirement for c-*src* activity in a similar fashion [11, 21]. On the other hand, mammary tumors co-expressing p53 and ErbB-2 could be interpreted as having lost one mechanism that controls cell proliferation and gained one that activates the malignant cellular potential. Overexpression of p53 and ErbB-2 may be related events or may be triggered by a common event that occurs during the earliest neoplastic steps [3, 22]. However, overexpression of the p53, c-*erb*B-2 and c-*yes* oncogenes indicates a high malignant potential of mammary tumors but whether there is a significant correlation between overexpression of these proteins and any prognostic factors in canine mammary tumors has not yet been established conclusively.

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Fig. 3. Immunohistochemistry of a simple adenoma with ErbB-2 expression. There is intense immunoreactivity at the cytoplasmic membrane of neoplastic cells (arrowheads). (SAB immunoperoxidase method, counterstained with Mayer's hematoxylin, × 400)

Fig. 4. Immunohistochemistry of an adenocarcinoma. The neoplastic cell membranes are outlined by a distinct ErbB-2 immunostaining pattern (arrowheads). (SAB immunoperoxidase method, counterstained with Mayer's hematoxylin, × 400)

Fig. 5. Immunohistochemistry of an adenocarcinoma with p53 expression. Note nuclear staining of malignant tumor cells (arrowheads). (SAB immunoperoxidase method, counterstained with Mayer's hematoxylin, ×400)

Fig. 6. p53 immunoreactivity in many malignant nuclei of solid carcinoma tumor cells (arrowheads). (SAB immunoperoxidase method, counterstained with Mayer's hematoxylin, × 400)

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