Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors

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We concentrated ourselves to evaluate the prognostic significance of the p53 gene mutations, its protein expression and MIB-1 index as a proliferative marker in canine mammary tumors. In the present study, a total of 20 cases were examined, among which there were 5 malignant mixed tumors, 4 mammary gland adenocarcinomas, 1 papillary adenocarcinoma, 8 benign mixed tumors and 2 mammary gland adenomas. Positive immunostaining for p53 with PAb240 antibody was found in 2 benign (20%) and 3 malignant (30%) tumors. However, PAb421 antibody did not give positive result at all. In Western blot analysis, the p53 expression in benign and malignant tumors was detected in 4 and 3 cases, respectively. p53 mutations were found in 6 cases out of the cases with detected p53 protein expression. The MIB-1 index in benign and malignant tumors were 17.6 ± 20.8% and 29.0 ± 27.2%, respectively and there was no significant difference between tumor types. There was a significant correlation between p53 mutations and p53 overexpression (correlation coefficient = 0.5, p < 0.05). In Kaplan-Meier survival analysis, the p53 index was associated with significantly shortened survival time (p < 0.01). In multivariate analysis, p53 overexpression was only an independent factor for indicator of worse prognosis in canine mammary tumors (p = 0.01). These results demonstrated that p53 gene mutations and protein overexpression using the PAb240 anti-p53 antibody were useful predictors of increased malignant potential and poor prognosis in canine mammary tumors.

Key words: canine, mutation, overexpression, p53, prognosis

Introduction

Canine mammary tumors account for half of all tumors in bitches and approximately 40-50% of them are considered malignant [2,3,24]. Effective treatment method with prompt accurate diagnostic procedure is the prime importance for this life threatening neoplasm. In surgical intervention, about 48% of dogs died or euthanized even within 1 year after their surgery due to recurrence or metastasis [10]. Despite of the intensive clinico-pathological investigation, a very little is known about the prognosis and causes of canine mammary tumor [2]. Precise clinical and pathologic strategies are subjected to numerous errors, and imaging methods are not very sensitive to initial tumor spread [21]. Therefore, accurate and additional prognostic aids are required to identify patients at high risk.

Recent advances in tumor biology have identified a number of markers that may form a basis for tumor stratification [7,10,26]. Numerous studies have been focused on the investigation of the significant role of the p53 tumor suppressor gene in the tumorigenesis of human and canine cancers. Mutations of the p53 gene are believed to be the most common genetic alteration in canine mammary tumors like other human and dog malignancies and many studies also indicated that p53 mutation is associated with tumor progression [11,16,17,30,33]. Mammary carcinomas in dogs have similarities of prevalence, metastasis and disease pattern compared with the breast cancer in human [27]. In humans, p53 gene mutations have been documented in breast cancer by numerous intensive studies [3,6]. These mutations have been detected in 15-34% of cases and have been considered an important indicator of poor prognosis and shortened survival rate [3,8]. Some abnormalities of the p53 gene have been documented in spontaneous thyroid carcinoma, oral papiloma, circumanal gland adenoma, osteosarcoma and lymphoma in dogs [5,14,18,19,32]. Our previous report with the data in the present study demonstrated that p53 mutations were in 7 out of 20 cases studied and 3 out of 4 dogs died of mammary carcinoma had a p53 mutation [15].

In the present study, the relationship among the clinical and histological parameters, the p53 gene mutations, its protein expression and MIB-1 index as a proliferative marker in canine mammary tumors was evaluated to get the prognostic markers.
Materials and Methods

Tumor specimens

Twenty female dogs were selected which were referred to the Veterinary Medical Teaching Hospital (VMTH), Seoul National University, for diagnosis and treatment. The individual basic data were described in our previous report [15]. Metastasis suspicions were solved by thoracic radiographs and ultrasonographs of liver, kidney and spleen before surgery. Each case was classified according to the clinical TNM staging of canine mammary tumors modified from the World Health Organization [24]. All patients underwent either by lumpectomy or mastectomy and none of the patients had experienced preoperative systemic chemotherapy or radiotherapy.

Mean follow-up period was 16 months (range, 2-38 months) and the last clinical assessment was used to determine final status. Survival time was defined as the time from tumor biopsy or excision to the time of death due to progression of disease or the last clinical assessment. Recurrence was defined as the occurrence of mammary tumor again after surgery at any stage or grade. Progression of the disease was considered at the death of the animal from cancer or remote lymph node or organs metastasis.

Tissue blocks of each tumor were frozen in liquid nitrogen immediately after surgical removal and stored at −70°C for DNA and protein extraction. Some adjacent sections were immediately fixed in 10% neutral buffered formalin and routinely processed for embedding in paraffin. Serial sections were cut 3 µm from each specimen block and prepared for immunohistochemistry and histopathology.

Mutational analysis

The mutational analysis of p53 was performed as described in our previous report [15].

Western blot analysis of anti-P53 antibody

Protein samples were prepared by homogenizing tumor specimens in buffer solution containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% TritonX-100, 1 µg/ml aprotinin and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF) using a Teflon pestle. They were then boiled at 100°C for 5 minutes. The lysates were sonicated and centrifuged at 12,000 rpm for 10 minutes. Supernatant protein concentrations of the lysates were measured using the BioRad protein assay kit (BioRad, Hercules, USA). Equal amounts of protein (20 µg) from each tissue sample were then boiled for 5 minutes and electrophoresed on a 10% SDS/polyacrylamide gel with prestained size markers (Color markers, Sigma, Saint Louis, USA). Following electrophoresis, proteins in the gels were transferred onto nitrocellulose membrane using Mini Trans-Blot® apparatus (BioRad, Hercules, USA). Relative protein concentration per lane and transfer efficiency were checked by staining nitrocellulose membranes with Ponceau S (Amresco Inc., Solon, USA). Membranes were blocked non-specific binding by incubating in blocking solution containing Tris-buffered saline (TBS)/0.05% Tween-20 (TBST) with 5% (w/v) skimmed milk overnight at 4°C. The blotted membrane was incubated in monoclonal mouse anti-human p53 protein antibody (PAb421, Oncogene™ research products, San Diego, CA, USA) diluted at 1 : 100 with blocking solution for one hour at room temperature and then rinsed three times for 5 minutes each with TBST, followed by anti-immunoglobulin G horseradish peroxidase conjugate secondary antibody (horseradish peroxidase conjugated goat anti-mouse IgG, Zymed Lab. Inc., So. San Francisco, CA, USA) diluted at 1 : 2000 with blocking solution. The membrane was washed three times for 5 minutes each with TBST and once for 5 minutes with TBS. Membranes were processed using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia biotech, Buckinghamshire, England) and autoradiography according to the manufacturers instructions.

Immunohistochemistry

The immunohistochemical study was performed using the antibodies against the p53 protein and MIB-1 on formalin-fixed, paraffin-embedded tissue specimens from initial tumors. PAb240 and PAb 421 (monoclonal antibody to p53 protein of mouse origin, 1:50 dilution, Oncogene™ research products), which recognize different epitopes of the p53 product, were used for the detection of overexpression of mutant p53 protein, and MIB-1 (monoclonal antibody to Ki-67 antigen of mouse origin, 1 : 50 dilution, Immunotech, Marseille, France) for the detection of Ki-67 antigen. Formalin-fixed sections were deparaffinized in two changes of xylene for five minutes each and rehydrated through sequential immersions in four changes of graded concentrations of ethanol. Sections were then rinsed in distilled water. For unmasking of nuclear antigen, tissue sections were boiled for six minutes using a microwavable pressure cooker on a citrate buffer (10 mM, pH 6.0), and were allowed to cool down gradually to the room temperature and then rinsed in PBS. In p53 staining, slides were digested in 0.1% porcine trypsin for 20 minutes at 37°C and rinsed three times with PBS. Endogenous peroxidase present within the tissue was inactivated by immersion of the slides in 3% hydrogen peroxide in methanol and the sections blocked with a protein blocker (Histostain SP kit, Zymed Lab. Inc., So. San Francisco, CA, USA). Each tissue section was incubated overnight at 4 with the appropriate primary antibody to p53 protein and MIB-1. Slides were rinsed three times in PBS, and then incubated for 30 minutes with biotinylated secondary antibody (Histostain SP kit, Zymed Lab. Inc.). PBS-washed sections were then incubated for 20 minutes in the streptavidin-
peroxidase conjugate solution (Histostain SP kit, Zymed Lab. Inc.) for detection of bound primary antibody. After washing in PBS three times, slides were incubated in 3, 3-diaminobenzidine solution. Color change was monitored on positive-control slides and was stopped by immersion in distilled water, and then briefly counterstained with hematoxylin only in MIB-1 immunostaining. Slides were dehydrated through ascending alcohol and xylene and then coverslip applied. All steps were carried out at room temperature in a humidified chamber unless otherwise indicated.

Formalin-fixed, paraffin-embedded human gastric cancer and oral squamous cell carcinoma tissue block were used as positive controls. Negative controls were provided by treating with non-immune serum, instead of the primary antibody. Histologically normal mammary gland tissue block served as negative tissue controls, and nonneoplastic tissue on each slide provided internal negative controls.

**Microscopic evaluation**

Light microscopic evaluation of immunohistochemically treated sections for positive nuclear staining was performed. The quality of each immunohistochemically stain was assessed by comparing the sections with an accompanying positive control slide.

A tumor sample was regarded as p53 positive if nuclear staining was clearly detected, but cytoplasmic staining alone was not recorded as positive. Positively staining was evaluated semi-quantitatively using a previously described system where 0 = no staining; 1 = <10%; 2 = 10-50%; and 3 = >50% of cells. Based upon previous reports [25,29], we considered tumors to be p53 positive by receiving 2 or 3 score.

Proliferative activity was examined by staining with an anti-Ki-67 specific antibody, MIB-1, and was evaluated separately in each case after counting at least 500 nuclei in 3-5 randomly selected high-power fields of the section (×400). Proliferation indexes were calculated as the percentage of cells with positive nuclear staining compared with the total nuclear area.

**Statistical analysis**

MIB-1 index was analysed with Mann-Whitney U test to determine whether differences per tumor type were significant. Correlation was estimated among clinicopathological parameters, p53 mutations, p53 index and MIB-1 index. Survival curves on each prognostic variables were computed using the Kaplan-Meier survival analysis and compared curves by log rank test. Multivariate Cox regression analysis was performed to determine the prognostic value of several parameters.

All statistical analyses were performed with software package SPSS (Release 8.0, SPSS inc.) and a P-value of <0.05 was considered as statistically significant.

**Results**

**Clinical features of the canine patients**

Histopathologic study revealed that there were 5 malignant mixed tumors (2 stage V, 1 stage IV, 2 stage III), 4 mammary gland adenocarcinomas (1 stage V, 3 stage IV), 1 papillary adenocarcinoma (1 stage I), 8 benign mixed tumors (2 stage IV, 3 stage II, 3 stage I) and 2 mammary gland adenoma (1 stage II, 1 stage I), 4 dogs with malignant tumors and 2 with benign tumors had palpably enlarged lymph nodes in axillary and inguinal region. It was found that 16 dogs were alive and 4 died. Local recurrence occurred in 4 dogs within 2, 6, 12 and 13 months after the first operation respectively, and further recurrence was found in a dog even after 1 month of re-excision.

**Identification of tumor-associated p53 gene alterations**

p53 gene alteration was found in 7 cases (35%) and their different mutational characteristics also identified. four missense and 1 non-sense mutations were detected in 10 malignant lesions (40%), and 2 missense and 1 silent mutations were found in 10 benign mammary tumors (30%). Among the 6 mis-sense mutations, 5 mutations were located in highly conserved domains II, III, IV and V. In a case, the codon change CGA → TGA results in the introduction of a stop codon at position 213 and another one showed the presence of a silent mutation. G:C → A:T transitions were detected in 5 mutations and transversions were shown in 3 dogs.

**Overexpression of p53 protein and MIB-1**

Various positive nuclear immunostaining was detected in each of the control sections of human gastric cancer and oral squamous cell carcinoma. Staining was not observed in negative controls treated with non-immune serum in place of the primary antibody.

Positive immunostaining for p53 protein with PAb240 antibody was found in 5 case (25%). The proportion of benign and malignant lesions stained for p53 are 20% and 30% respectively (Fig. 1b, 2b). However, PAb421 antibody did not give positive result at all. There was a significant correlation between p53 mutations and p53 overexpression (correlation coefficient = 0.50, p < 0.05, Table 1).

In Western blot analysis, the p53 protein expression in benign and malignant tumors was detected in 4 and 3 cases, respectively (Fig. 2). p53 gene mutations were found in 6 cases out of the cases with detected p53 protein expression.

The MIB-1 positive range was from 2% to 75% (23.3 ± 24.3%). The MIB-1 index in benign and malignant tumors were 17.6 ± 20.8% and 29.0 ± 27.2% (Fig. 1c, and 2c). There was no significant difference in the MIB-1 index between tumor types.
In Kaplan-Meier survival analysis, the p53 index was associated with significantly shortened survival time (Fig. 3, \( p < 0.01 \)). The results of multivariate analysis for determining the prognostic value of several parameters are shown in Table 2. P53 overexpression was only an independent factor for indicator of worse prognosis in canine mammary tumors \( (p = 0.01) \).

**Discussion**

In the present study, p53 immunohistochemical expression by using PAb240 anti-human p53 antibody is found in 25% of the canine mammary tumors. Similar expression rate was reported by other investigators [9,28,  

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**Fig. 1.** Photomicrographs of a section of the case with stage II mammary gland adenoma (1a, 1b, 1c) and of a section of the case with stage V malignant mixed tumor (2a, 2b, 2c) stained with hematoxylin and eosin (a), immunohistochemically for p53 with an anti-p53 antibody (PAb240, Oncogene) (b) and MIB-1 with an anti-Ki-67 antibody (MIB-1, Immunotech) (c). (1a) Note well-differentiated and well-capsulated neoplastic cells. H&E stain, \( \times 200 \). (1b) Note weak p53 nuclear positive immunostaining of several tumor cells. No counterstain, \( \times 200 \). (1c) Note moderate proliferative activity of several neoplastic cells expressed as diffuse MIB-1 immunostaining. Hematoxylin counterstain, \( \times 200 \); (2a) Note pleomorphic tumor cells with a moderate amount of cytoplasm and hyperchromatic 2 to 3 nuclei. H&E stain, \( \times 200 \). (2b) Note diffuse strong p53 nuclear positive immunostaining of several tumor cells. No counterstain, \( \times 200 \). (2c) Note high proliferative activity of several neoplastic cells expressed as diffuse MIB-1 immunostaining. Hematoxylin counterstain, \( \times 200 \).
p53 as a prognostic factor in cmt 67

The PAb240 antibody used in this study has an epitope within amino acid residues 371-380 of human p53 and is able to stain tumor cells with p53 mis-sense mutations. In many other studies, immunoreactivity of the canine p53 protein towards CM-1 (rabbit anti-human p53 polyclonal antibody), PAb240 (mouse anti-human p53 monoclonal antibody), BP53-12 and PAb122 (mouse anti-human p53 monoclonal antibody), which recognize different epitopes of the p53 product, has been found in various canine neoplasms by immunohistochemical analysis [1,9,12,25,31,35]. Veldhoen and Milner [31] suggested that canine p53 protein had a strong reactivity in an immunoprecipitation assay towards monoclonal anti-human antibody, PAb421. In order to define the immunoreactivity of canine p53 further, PAb421 antibody was used in this study by immunohistochemistry. However, PAb421 antibody did not give positive result at all. Albaric et al. [1] and Haga et al. [12] suggested that p53 positive result was able to alter according to different p53 antibodies and especially Ab-7 and DO-7 anti-human p53 antibodies did not react in canine tumors. This demonstrated that there might be local differences in the nature and organization of amino acid residues on the surface of the canine p53 molecule when compared to human p53 proteins.

Multivariate regression analysis and Kaplan-Meier survival analysis in the present study revealed that the p53 overexpression index is an independent risk factor for increased recurrence and death from these tumors and significantly shortened the survival time. Similarly it has been suggested that alterations in p53 expression correlated with highly aggressive tumor behavior as a promising new parameter to evaluate the cellular biology and prognosis of human mammary ductal carcinoma [22,25]. P53 expression tends to be more frequent in phyllodes tumors with higher malignant potential [29]. However, reported elsewhere immunohistochemistry for p53 expression is not a suitable prognostic markers in canine mammary carcinoma and female breast cancer [20,34].

Positive staining of p53 protein was detected in two benign mammary tumors accompanied by increased index of MIB-1 in this study. A recent study by Rohan et al. [23] concluded that p53 staining in benign breast biopsies was associated with an increased risk of future breast cancer. Thus, p53 protein levels of wild type or mutant protein may be associated with the subsequent development of canine mammary and human breast cancer Many investigations have been focused on the role of immunohistochemical overexpression in predicting p53 mutation [11]. Done et al. [6] concluded that p53 inactivation occurred prior to invasion in breast carcinogenesis, with mutations being uniformly identified in ductal carcinoma in situ associated with p53-mutated invasive carcinomas.

Immunohistochemical analysis of MIB-1, as a proliferative marker is a good approach for evaluation of the growth fraction [4,13]. MIB-1 is a monoclonal antibody against recombinant parts of the Ki-67 antigen and true Ki-67 equivalents [4]. Sarli et al. [26] suggested that MIB-1 index revealed a significant association with prognosis in canine malignant mammary tumors. The MIB-1 immunostaining found in this study tended to be more frequent in malignant mammary tumors, but it was not

| Table 1. Correlation coefficient rates between clinicopathological parameters, p53 mutations, p53 index and MIB-1 index |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Stage           | Tumor type      | P53 mutations  | P53 index       | MIB-1 index     |
|                 |                 |                 |                 |                 |
| 1               | 0.677           | 1               | 0.196           | 0.501           |
| Tumor type      | 0.105           | 0.153           | 0.048           | 0.226           |
| P53 mutations   | 0.501           | 1               | 0.429           | 0.397           |
| P53 index       |                 |                 |                 |                 |
| MIB-1 index     |                 |                 |                 |                 |

*p < 0.01, **p < 0.05

Table 2. Multivariate analysis of clinicopathological factors, p53 mutations, p53 overexpression and MIB-1 index.

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<td>P53 mutations</td>
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<td>P53 overexpression</td>
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<td>MIB-1 index</td>
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N.S.: not significant

Fig. 2. P53 protein expression in benign (a) and malignant (b) mammary tumors by Western blot.
The present study suggested that p53 gene mutations and protein overexpression using the PAb240 anti-p53 antibody were useful predictors of increased malignant potential and worse prognosis in canine mammary tumors.

References