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 precasted 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-