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Mutations of *p53* Tumor Suppressor Gene in Spontaneous Canine Mammary Tumors

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Abstract

Mutation of the *p53* tumor suppressor gene has been related in the pathogenesis of numerous human and canine cancers, including breast cancers and mammary tumors. We have investigated exons 5-8 of the *p53* gene for mutations in 20 spontaneous canine mammary tumors using polymerase chain reaction (PCR) with direct sequence analysis to evaluate the role of this gene in canine mammary tumorigenesis and analyzed to compare with other clinicopathological parameters including age, histology, stage, recurrence and death from tumor. Four missense (one case had two missense mutations) and one nonsense mutations were detected in 10 malignant lesions (40%), and two missense and one silent mutations were found in 10 benign mammary tumors (30%). Five of the missense mutations were located in highly conserved domains II, III, IV and V. After a follow-up period, four dogs showed a progression and three of these patients revealed death from mammary carcinoma with *p53* mutation. These results demonstrated that the *p53* gene mutations might be involved in the development of canine mammary tumors and contribute to the prognostic status in canine mammary carcinomas.

Key words : dog, mammary tumor, *p53* gene mutation

Introduction

Recent advances in tumor biology have identified a number of markers that may form a basis for tumor stratification [7,10,25]. Especially, 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. The *p53* gene mutates most commonly in canine and human cancers and encodes 381 and 393 amino acid nucleophosphoprotein, respectively [4,15]. Its product, wild type *p53* protein is a 53-kd nuclear phosphoprotein which acts as a negative transcriptional factor with diverse functions including the regulation of cell cycle and interactions with other transcription factors [3]. In addition, *p53* protein may retain cells in G1 phase to allow DNA repair for occurrence or induction of programmed cell death (apoptosis) in cases of irreversible damage [33]. It is believed that *p53* protects the cells against mutations by ensuring genomic stability, but the damage of *p53* gene may lead to a loss of its growth-inhibitory functions and contribute to uncontrolled cell cycle by several mechanisms [9,12].

Mammary tumor is one of the most common neoplasms in female dogs and women. Canine mammary tumors may account for half of all tumors in bitches and approximately 40-50% of them are considered malignant [1,2,24]. Mammary carcinomas in dogs have similarities with the breast cancer in human beings, including the high prevalence of adenocarcinomas, frequency of metastases, and progressive disease [26]. In humans, *p53* gene mutations have been documented in breast cancer by numerous intensive studies [2,6]. These mutations have been detected in 15-34% of cases analyzed and have been considered an important indicator of poor prognosis and shortened survival rate [2,8]. Mutations of the *p53* gene are believed to be the most common genetic alteration in canine mammary tumors like other human and dog malignancies [11,15,17,28,31]. Some abnormalities of the *p53* gene have been documented in spontaneous thyroid carcinoma, oral papilloma, circumanal gland adenoma, osteosarcoma, and lymphoma [5,13,19,20,30]. However, there are limited researches related to *p53* gene mutations in canine neoplasms.

We have investigated exons 5-8 of the *p53* gene for mutations in 20 spontaneous canine mammary tumors using polymerase chain reaction (PCR) with direct sequence analysis to evaluate the role of this gene in canine mammary tumorigenesis and analyzed to compare with other clinicopathological parameters including age, histology, stage, recurrence and death from tumor.

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Materials and Methods

Dogs with canine mammary tumors

Twenty female dogs referred to the Veterinary Medical Teaching Hospital (VMTH), Seoul National University, for diagnosis and treatment of primary mammary tumors were included in this study. The main clinicopathological parameters of the tumors are presented in Table 1. The mean age of the dogs was 9.1±1.52 years (range, 7-13 years) and two were mixed breeds and eighteen were purebred dogs (6 Malteses, 5 Poodles, 5 Yorkshire Terriers, 1 Australian Terrier and 1 Shih Tzu). To identify distant metastases, thoracic radiographs and ultrasonographs of the liver, kidney and spleen were obtained 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 lumpectomy or mastectomy, and none of the patient had experienced preoperative systemic chemotherapy or radiotherapy.

Average follow-up period was 16 months (range, 2-38 months). 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 metastasis of distant lymph node or organs.

Tumor specimens

Tissue blocks of tumor specimen were frozen in liquid nitrogen immediately after surgical removal and stored at -70℃ for DNA extraction. Some adjacent sections were immediately fixed in 10% neutral buffered formalin and routinely processed for embedding in paraffin. Serial sections were cut 3 ㎛ from each specimen and prepared for routine histopathologic examination.

Mutational analysis

Genomic DNA was extracted from the frozen tumor specimens using a DNAzol reagent (DNAzol®, GIBCOTM Invitrogen Co., Grand Island, USA), modified technique of guanidine salts extractions.

PCR oligonucleotides for amplification of the *p53* fragments and PCR condition were designed on the basis of previously published sequencing data [13,14,23] and used to generate approximately a 1.2 kb fragment including exon 5, exon 6, exon 7 and exon 8 fragments, the highly conserved regions of canine *p53* gene. 0.1 μM of primers Cp53S (5′-TGA CCT GTC CAT CTG TCC TT-3′) and Cp53R (5′-ATC ATG CCT GAT GCT CAA CC-3′) were mixed with 200 ng of canine genomic DNA, 1.5 mM MgCl2, 200 μM dNTP's, 1 unit *Taq*-polymerase (Core Taq), and 10× PCR buffer, in a final volume of 50 μl. PCR was carried out for 35 cycles of

Table 1. Histologic diagnosis, TNM stage, survival time, type of death and *p53* mutation in twenty canine mammary tumors

Tumor sample	Age	Diagnosis	TNM stage	Survial(month)*	Type of death**	*P53* mutation
CMT01	9	Adenoma	II	15+ .	A	+
CMT02	9	Benign mixed tumor	I	17+ .	A	
CMT03	8	Benign mixed tumor	II	16+ (R)	A	+
CMT04	8	Benign mixed tumor	II	19+ .	A	+
CMT05	11	Benign mixed tumor	I	14 .	A	
CMT06	8	Benign mixed tumor	II	14+ .	A	
CMT07	9	Benign mixed tumor	IV	38+ (R)	A	
CMT08	10	Benign mixed tumor	IV	16+ .	A	
CMT09	7	Benign mixed tumor	I	11+ .	A	
CMT10	9	Adenoma	I	10+ .	A	
CMT11	8	Adenocarcinoma	IV	<3 (P)	Y	+
CMT12	10	Papillary adenocarcinoma	II	33+ (R)	A	
CMT13	9	Adenocarcnioma	IV	33+ .	A	
CMT14	12	Malignant mixed tumor	IV	3 (R)	E	
CMT15	9	Adenocarcnioma]	V	21 (R)	A	
CMT16	10	Malignant mixed tumor	III	21+ .	A	
CMT17	10	Adenocarcnioma	IV	15+ .	A	
CMT18	10	Malignant mixed tumor	V	<12 (P)	E	+
CMT19	7	Malignant mixed tumor	III	12+ .	A	
CMT20	11	Malignant mixed tumor	V	<2 (P)	E	+

*P = progression; R = recurrence.

**Y = death from cancer; A = alive at last report; E = by euthanasia.

denaturation (94℃, 30 sec), annealing (58℃, 30 sec), and polymerization (72℃, 2 min) steps, followed by a final extension step for 10 min at 72℃. The obtained PCR product was run on a 1.5% agarose gel electrophoresis to check the specificity of the reaction under UV light and photographed with a Polaroid camera.

The PCR products were gel-purified in a 1.5% agarose gel using CONCERT™ gel extraction systems (GIBCOTM Invitrogen Co., Grand Island, USA), and directly cloned into the plasmid with TOPO TA cloning® (Invitrogen, Carlsbad, USA) kits. PCR products ligated into pCR®2.1-TOPO® vector of the TA cloning kit, and transformed the recombinant plasmid into TOP10 competent *Escherichia coli* (Invitrogen, Carlsbad, USA). Each transformed bacteria was plated onto Luria-Bertani (LB) agar plates containing ampicillin (50 μg /ml) and incubated overnight at 37 ℃. Picked 10 colonies and cultured them overnight at 37 ℃ with vigorous shaking in LB medium containing 50 μg/ml of ampicillin. Plasmid DNAs were extracted with a Qiagen plasmid mini kit (QIAGEN, Valencia, USA). Double stranded DNA was sequenced according to the dideoxy chain termination method using an Auto Read Sequencing Kit (ALFwin Sequence Analyser 2.00, Amersham Pharmacia Biotech, USA). Sequence analysis was performed at least twice, using independently amplified and subcloned PCR products to exclude PCR artifacts.

The homology of nucleotide sequences of PCR products which were obtained by sequencing analyzer were calculated by BLAST program (NCBIs sequence similarity search tool, http://www.ncbi.nlm.nih.gov/BLAST).

Results

The *p53* gene alterations were found in seven of the twenty cases studied (35%) and the summary of the mutations identified is shown in table 2. Four missense (One malignant case (CMT18) had two missense mutations on exon 7 and 8) and one nonsense mutations were detected in ten malignant lesions (40%), and two missense and one silent mutations were found in ten benign mammary tumors (30%). Among the six missense mutations, five mutations were located in highly conserved domains II, III, IV and V. In one case (CMT20), the codon change CGA → TGA results in the introduction of a stop codon at position 213 and another one (CMT04) showed the presence of a silent mutation. G:C → A:T transitions were detected in five mutations and transversions were shown in three dogs.

After a follow-up period, four dogs showed a recurrence and four dogs progressed, and three of four patients that showed a progression revealed death from mammary carcinoma accompanied by *p53* mutation.

Discussion

The *p53* tumor suppressor protein plays a central role in the regulation of cell proliferation, genomic stability, and programmed cell death [28,29]. But the *p53* gene mutation leads to an amino acid substitution in the protein and may contribute to deregulated cell growth and tumor resistance to chemotherapy [29]. *p53* mutations are the most common genetic alterations found in human tumors, including cancers of the breast, lung, colon, osteosarcoma and others [16,27]. And the investigations on the role of *p53* mutation in the carcinogenesis of spontaneous canine tumors have been performed [5,13,17,19,20]. This suggests that the role of wild type *p53* protein in preventing tumor formation and progression may be similar in both humans and dogs.

In the present study, *p53* gene mutation was demonstrated in seven cases out of twenty canine mammary tumors and six missense mutations were found in five dogs. This result is similar to that of previous report of Devilee *et al*. [5], where the great majority of the mutations in *p53* gene were missense. Recently, it is documented that similar *p53* gene mutations in canine mammary tumors have been identified. These *p53* mutations are located at human codon numbers 21, 22, 24, 82, 102, 116, 138, 175, 176, 236, 245, 249 within exon two, four, five, and seven [17,18,28,31]. And also nonsense, splicing, and frameshift mutations in exon 4, 5, 6, and 7 of the *p53* gene have been detected in canine mammary

Table 2. Mutations in *p53* exons 5-8 identified in canine mammary tumors

Tumor sample	Breed	Exon	Codon*	(CD**)	Mutation	Amino acid substitution
CMT01	Yorkshier terrier	7	245	(Ⅳ)	G<u>G</u>C → G<u>C</u>C	Gly → Ala
CMT03	Maltese	5	173	(Ⅲ)	<u>G</u>TG → <u>T</u>TG	Val → Leu
CMT04	Yorkshier terrier	8	305	(n)	AA<u>G</u> → AA<u>A</u>	Silent
CMT11	Maltese	8	285	(Ⅴ)	<u>C</u>CT → <u>T</u>CT	Pro → Ser
CMT16	Maltese	5	129	(Ⅱ)	<u>C</u>TC → <u>T</u>TC	Leu → Phe
CMT18	Maltese	7	248	(Ⅳ)	C<u>G</u>G → C<u>A</u>G	Arg → Gln
		8	297	(n)	C<u>C</u>T → C<u>G</u>T	Pro → Arg
CMT20	Poodle	6	213	(n)	<u>C</u>GA → <u>T</u>GA	Arg → Stop

*Corresponding to human *p53* gene

**Conserved domain, corresponding to human *p53*

tumors [4]. These studies indicate that *p53* mutation is associated with tumor progression. In a variety of human cancers, more than 90% of missense mutations in *p53* span highly conserved domains, DNA binding domain (codon 102-292) which is localized between exons 5-8, and this part is well known for harboring "hot spots" in canine and human tumors [11]. We found five missense mutations in these regions also.

The *p53* missense point mutations reported in the CMT01, CMT18 and CMT20 dogs correspond to the previously identified *p53* gene of various canine and human tumors [13,28,30]. Two of these three mutations were found at codons 245 and 248, two of six codons involved with over 40% of *p53* gene mutations identified in various human tumors. And the other one (CMT20) displayed a nonsense point mutation at codon 213 of exon 6. This point mutation (CGA → TGA) results in a substitution from arginine to stop codon and may cause premature termination of protein synthesis at the mutant codon. This is likely to abolish protein function, because only the front part of the protein may be produced in the mutant cell. The case (CMT04) with benign mixed tumor was found to have a point mutation in codon 305 (canine codon number 293) of exon 8. However, this mutation is silent and it does not appear to play a role in the development of the tumor.

Three *p53* mutations identified were found in benign tumors. These tumors were diagnosed histologically as a mammary gland adenoma and mixed mammary tumors. These mutations in benign lesion have also been reported in the other canine and human tumors [21,22]. Thus, *p53* mutations may sometimes occur at a histological section at the early stage of development and may indicate a greater propensity of the lesion to progress, although further studies are needed to discuss this.

Five of the eight mutations observed were G:C → A:T transitions, and three were G:C → T:A transversions. In human cancers, G:C → A:T transitions are the major point mutations (47%) of all *p53* gene identified in human cancers [13]. But the transversions in which a purine is replaced by a pyrimidine or vice versa are rare.

In this study, three of four dogs died of mammary carcinoma were found to have a *p53* mutation. Because inactivation of *p53* is more common in advanced tumors, results in increased proliferation and resistance to apotosis, and may facilitate metastatic spread through angiogenesis, inactivation of *p53* should be associated with a worse prognosis [8]. Wakui *et al*. [32] suggested that the *p53* mutations might contribute to the prognostic status in canine mammary carcinomas.

In conclusion, these results demonstrated that the *p53* gene mutations might be involved in the development of canine mammary tumors and contribute to the prognostic status in canine mammary carcinomas.

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