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Original article

IDENTIFICATION OF *P53* GENE ALTERATIONS IN CANINE MAMMARY TUMOURS USING POLYMERASE CHAIN REACTION AND DIRECT SEQUENCE ANALYSIS

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Summary

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Mammary tumours are mentioned as the most common tumours in female dogs and approximately half of them are detected malignant. *p53* gene mutations are demonstrated to be the most common genetic alteration in canine mammary tumours. The present study was conducted to evaluate exon-1 of *p53* gene mutations in tissue samples of canine mammary tumours by PCR and direct sequence analysis. After histopathological confirmation of the tissue sections by haematoxylin and eosin staining (10/26), deparaffinised samples were used for DNA extraction by silica gel method. Subsequently, *p53* exon 1 was amplified through PCR assay using specific oligo nucleotide primers designed according to the canine DNA sequence available online. Microscopically, 10 out of 26 suspected tissue samples were recognised as malignant mammary gland tumours with various grades of malignancy. Surprisingly, one insertion of mutation was found in exon 1 of all examined samples corresponding to a sequence comprising 27 amino acids, between amino acids 30 to 57 in the *p53* protein. Taken together, it seems that alteration of exon 1 *p53* gene may lead to malignancy behaviour, poor prognosis and short survival time in dogs with mammary carcinomas.

Key words: dog, exon 1, mammary tumour, mutation, *p53* gene

INTRODUCTION

The *p53* gene which encodes *p53* nuclear phosphoprotein acts as a tumour suppressor gene and has a pivotal role in the tumourigenesis of different organs through the modulation of cell growth and proliferation, apoptosis and genomic stability (Hainaut *et al.*, 1997). Indeed, *p53* protein can sustain cells in G1 phase to let DNA repair and induction of apoptosis in genomic irreversible injuries (Yonish-Rouach *et al.*, 1991). When *P53* is phosphorylated at serine 15 and 20 by ATM and checkpoint 2, respectively, it displays critical function in DNA repair. On the other hand, if phosphorylation occurs at serine 46 by other phosphokinases, it will influence programmed cell death (Mayo *et al.*, 2005; Feng *et al.*, 2006; Smeenk *et al.*, 2011). Moreover, it is considered that *p53* guards the cells against mutations via ensuring genomic stability (Gamblin *et al.*, 1997). Therefore, biological disorder of *p53* gene, protein or pathways can impair the mentioned cellular mechanisms. In this regard, *p53* abnormalities are demonstrated in various human malignancies such as breast cancer (Nakopoulou *et al.*, 1996), ovarian carcinoma (Dong *et al.*, 1997; Shahin *et al.*, 2000), prostate carcinoma (Cheng *et al.*, 1999), renal adenocarcinoma (Lipponen *et al.*, 1994), gastric cancer (Igarashi *et al.*, 1999) and colorectal cancer (Kaserer *et al.*, 2000). Similarly, alterations of *p53* gene have been identified in different canine tumours such as mammary gland tumours (Muto *et al.*, 2000; Lee & Kweon, 2002; Lee *et al.*, 2004), lymphoma (Veldhoen *et al.*, 1998; Koshino *et al.*, 2016) and osteosarcoma (Loukopoulos *et al.*, 2003). Mammary tumour is considered the most common neoplasms in female dogs which accounted for 50% of all tumours in bitches and about 40–50% of them are malignant (Rutteman *et al.*, 2001). Mutations of the *p53* gene are reported to be the most common genetic alteration in canine mammary tumours (Veldhoen *et al.*, 1999). Previous studies have investigated some alterations of exons 4–8 of *p53* gene for canine benign and malignant mammary tumours. The present study was carried out to detect exon-1 of *P53* gene mutations in canine mammary tumours via PCR and direct sequence analysis.

MATERIALS AND METHODS

Sample collection

Over a 6-month period from March to August 2011, 26 female dogs referred to the Hospital of Faculty of Veterinary Medicine, University of Tabriz, Tabriz,

Table 1. Histopathological diagnosis associated with mutations of exon-1 of *P53* in canine mammary tumours (10 out of 26 samples)

Pathological diagnosis	Number of samples	Grade	Age	Breed	Survival (months)
Spindle cell carcinoma	2/10	G2	2/4	Mixed Terrier/ Terrier	6/6
Tubulo-papillary* carcinoma	2/10	G1	5/9	German Shepherd/Xianlou	19/22
Tubulo-papillary* carcinoma	1/10	G2	6	Terrier	24
Carcinosarcoma (mixed mammary tumour)	2/10	G2	11/11	Spitz/Poodle	3/4
Simple tubular carcinoma	1/10	G2	12	Yorkshire Terrier	6
Complex carcinoma	1/10	G3	2	Rottweiler	6
SCC	1/10	G2	7	Dobermann	3

*semi total mastectomy was performed in tubulo-papillary carcinoma.

Iran, on the occasion of mammary region macroscopic lesion suspected to be mammary gland tumours, were used for the present study.

As is shown in Table 1, the dogs were classified in three age groups (≤3, 3–6, and ≥6 years old). Immediately after local surgery, tissue samples of about 1–3 cm in thickness from the removed masses were considered for histopathological investigation. The taken specimens were fixed by 10% neutral buffered formalin, then dehydrated in graded ethanol, and embedded in paraffin. Finally, sections with 5 μm thickness were stained routinely by haematoxylin and eosin and studied by light microscopy (OLYMPUS-CH30, Japan).

Definitive diagnosis and grading of the cancers were performed according to criteria presented previously (Goldschmidt *et al.*, 2017).

Molecular assay

DNA extraction: After deparaffinisation with xylenes and ethanol, extraction of DNA from tissues were performed according to the manufacturer's instructions provided using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). Approximately 25 mg of tissue samples were moved to a sterile 1.5 mL microcentrifuge tube consisting of 180 μL ATL buffer and 20 μL Proteinase K and incubated in a water bath at 55 °C to disperse the sample for 20 min. After that, 4 μL of RNase A (100 mg/mL) was added to the mixture, incubated for 2 min at room temperature and then mixed for 15 s by vortexing. A total of 200 μL ATL buffer was added to the sample, mixed thoroughly by vortexing and incubated at 70 °C for 10 min. The mixture was then added with 200 μL ethanol (96–100%) and mixed by vortexing to yield a homogenous solution. The homogenous solution was pipetted into the DNeasy® mini column sitting in a 2 mL collection tube and centrifuged at 12,000 *g* for 1 min. The DNA bound to the column was washed in two centrifugation steps using 500 μL AW1 buffer and AW2 buffer to improve the purity of the eluted DNA. The purified DNA was then eluted from the column in 100 μL AE buffer and stored at 4 °C until further use.

PCR assay: p53 exon 1 was amplified by PCR. Primers set used in this study were: forward: 3'-CAAGGTGAGGCT GATGAC-5' and reverse: 3'-TCGCCT KTCAATGCCAAG-5'). The reaction mixture (25 μL) contained $MgCl_2$ (4 mM), 1 mM dNTP mix (Bioline, UK), 2 mM of each primer, 0.2 U Taq (Bioline, UK), and 2 μL of extracted DNA. The thermal protocol comprised 5 min at 95 ^{o}C for initial DNA denaturation, followed by 30 cycles programmed as followed: 95 ^{o}C for 30 s (denaturation step), 60 ^{o}C for one minute (annealing step), and 72 ^{o}C for 30 s (extension step) and 10 min final extension at 72 ^{o}C. The products of traditional PCR were separated on a 2 percent (w/w) agarose gel, in TAE buffer and stained with Gel Red (Sigma, USA). A 50 bp DNA ladder (Fermentase, Ukraine) was used as a size marker. The gel photos were recorded by a Syngene gel documentation system.

Sequencing: Following PCR, the product was sliced from the agarose gel. All extra primers, salts and dNTPs were removed by using a gel purification kit (QIAquick Gel Extraction kit). Sequencing was performed by ABI3730XL sequencer machine (Macrogen, South Korea). DNA sequences were analysed by Bio Edit software version 7.2.1.

RESULTS

Histopathological findings

At microscopical examinations of the prepared tissue sections, ten out of 26 (38.6%) suspected samples were investigated as mammary gland tumours with different histologic classification presented in Table 1 and Fig. 1. Interestingly, all of the tumours showed malignant characteristics on the basis of previously mentioned criteria including formation of tubules, variation in nuclear size and shape, hyperchromatic nucleus, presence of nucleoli and intermediate to high mitotic index indicated in Table 1 as G1, G2 and G3 (low, intermediate and high grade of malignancy, respectively).

Molecular evaluations

P53 exon 1 was successfully amplified by PCR and specific primers. Through comparing the results of sequencing in our study with the data available in genomic resources of NCBI (accession number NC_000017.10), the presence of insertion mutations in exon 1 (Table 2) was revealed in all cases of canine mammary gland carcinoma in the present study regardless of pathologic pattern detected in the carcinoma.

Table 2. Insertion mutations found in exon 1 of p53 in canine mammary gland carcinoma.

Number of samples	Codon position	Mutations
2/10	7687588	GGC→GGTC
3/10	7687561	TGC→TAGC
1/10	7687552	CCT→CATCT
1/10	7687540	TGG→TGCCG
1/10	7687481	TCT→TAGCT
1/10	7687478	AGA→AGGA
1/10	7687469	GTC→GTTC

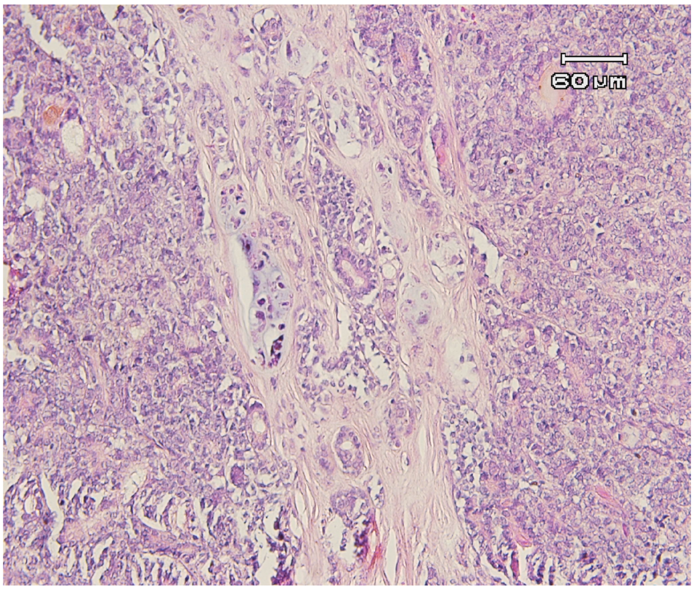

Fig. 1. Mixed mammary gland tumour, dog. Carcinoma cells surrounded by neoplastic spindle cells with foci of chondroid differentiation (H&E, bar=60 µm).

DISCUSSION

In the current study, all of the ten diagnosed mammary tumours were malignant and mutation of exon 1 *p53* gene was indicated using molecular method in all of the 10 carcinomas. Previously, similar studies on canine mammary tumours demonstrated that the alteration of exons 5–8 in the *p53* tumour suppressor gene (which is well known as *hot spots* in canine and human tumours) in 11% and 40% of the malignant and 20% and 30% of benign tumours (Muto *et al.*, 2000; Lee and Kweon, 2002). Additionally, overexpression of *p53* have also been reported by immunohistochemistry assay in other canine tumours such as osteosarcomas (Sagartz *et al.*, 1996), colorectal tumours (McEntee & Brenneman, 1999; Wolf *et al.*, 1997), cutaneous tumours (Ginn *et al.*, 2000; Jaff *et al.*, 2000), seminomas and Sertoli-cell tumours (Inoue & Wada, 2000). These findings generally indicate the important role of *p53* gene alterations in incidence of canine tumours like those in men. Importantly, most of the mutations investigated in tumours in dogs are point mutations located in the conserved domains of *p53* gene which were frequently accompanied with the single nucleotide insertions (Lee *et al.*, 2004; Tomiyasu *et al.*, 2010; Koshino *et al.*, 2016). Also, nonsense, splicing and frameshift mutations have been particularly indicated in exon 4, 5, 6, and 7 of the *p53* gene of canine mammary tumours (Chuu *et al.*, 1998). In the present study, however, a continuous 27-amino acids sequence was found surprisingly between 30-57 amino acids sequence in the *p53* protein. Anyway, it was previously proposed that the *p53* gene aberration leads to an amino acid displacement in the protein and may contribute to dysregulated cell growth and chemotherapy resistance in tumours (Veldhoen & Milner, 1998).

In the present study, because of the small numbers of the detected cancerous dogs, any correlation between the age, grade and breed of the affected animals with the identified *P53* gene mutation could not made. Therefore, further study with larger number of dogs including both malignant and benign tumours is required to approve the relationship of the *p53* gene aberration, animal history and prognosis. In this regard, some previous studies determined that *p53* expression is not prognostically convenient in canine colorectal cancers (Wolf *et al.*, 1997) and cutaneous mast cell tumours (Jaff *et al.*, 2000). However, another study presented the association of the *p53* expression with tumour grade and site on cutaneous mast cell tumours (Ginn *et al.*, 2000). In addition, another study demonstrated a direct correlation between the *p53* index and histopathologic characteristics of canine osteosarcomas (Loukopoulos *et al.*, 2003). More recently, it was also reported that the *p53* mutation can be investigated as a new prognostic tool in canine lymphoma (Koshino *et al.*, 2016). Accordingly, regarding to the overall survival found in the evaluated samples in this study, it seems that the alteration of exon 1 of *p53* gene may lead to malignancy behaviour, poor prognosis and short survival time in canine mammary carcinomas.

In conclusion, it was clarified that alteration of exon 1 *p53* gene like that of exon 4–8 may be related to poor prognosis, malignancy behaviour and reduced overall survival time in dogs with mammary carcinomas regardless of the pathologic pattern, breed and animal age range.

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