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Mutations of *p53* gene in canine sweat gland carcinomas probably associated with UV radiation

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Abstract

Introduction: Apocrine sweat gland carcinomas (ASGCs) are rare malignant skin tumours in dogs and humans. The literature published so far focuses mostly on the clinico-epidemiological aspect of these tumours, but little is known about their pathogenesis. In this study we aimed to determine whether the *p53* gene is involved in the carcinogenesis of the apocrine sweat gland in dogs and whether ultraviolet radiation (UV) is related to it. **Material and Methods:** Forty canine ASGCs were submitted to laser capture microdissection to isolate neoplastic cells, from which DNA was subsequently extracted. PCR amplification and sequencing of *p53* exons 2–8 was then performed, followed by computer analysis of the obtained sequences. **Results:** Sixteen mutations within the *p53* gene were found in 13 tumours. The mutations involved C → T, T → C, G → A, and CC → TT transitions, C → G transversion and adenine deletion, which are gene alteration types known to be related to UV radiation in the process of skin carcinogenesis in humans. Six of the thirteen tumour cases displayed the C → T transitions in the same location in exon 4 and three of the thirteen cases displayed T → C in the same location in exon 5. **Conclusion:** The results of the present study indicate both the participation of the *p53* gene and the influence of UV radiation in the formation of ASGCs in dogs.

Keywords: dog, apocrine sweat gland carcinoma, *p53* gene, mutations, UVR.

Introduction

Apocrine sweat gland carcinomas (ASGCs) are rare malignant skin tumours which represent approximately 0.7% to 2.2% of all skin neoplasms diagnosed in dogs (3, 4, 29, 56). Individual cases of this type of tumour were reported in cats (14, 16, 50), cattle (54, 61), horses (2, 11) and a rabbit (41). ASGCs are also very rare in humans, with an incidence rate estimated to range from 0.0049 to 0.0173 per 100,000 patients per year (17). In dogs, these cancers have been found to occur at older age (from 6 to 17 years) in both sexes, mainly in golden retrievers, mixed breeds and German shepherds (29, 56). Tumours can grow anywhere on the body, but most frequently on the head, neck, thorax and limbs (23, 29, 36, 56). They are usually aggressive, with a tendency to local recurrence and metastasis to the regional lymph nodes, lungs, liver, bone and bone marrow *via* lymphatic and intravascular invasion both in humans (4, 36, 48, 65) and dogs (3, 19, 22, 42, 56). The available literature describing canine ASGCs provides reports which focus mostly on the morphological appearance and epidemiological data, whereas little is known about the aetiology and molecular alterations underlying the development of these cancers.

Carcinogenesis has been described as a multi-stage process and the result of many interrelated genetic changes associated with the influence of one or multiple specific genotoxic aetiological factors. The pathogenesis of approximately 50% of human skin cancer cases has been associated with ultraviolet radiation (UVR), which has been found to cause characteristic alterations within the *p53* tumour suppressor gene (12). These alterations have been detected in normal sun-exposed skin (1, 5, 34), in precancerous lesions such as actinic keratosis and Bowen's disease, in squamous and basal cell carcinomas

(25) and, less frequently, in melanomas. Therefore, UVR is considered to be an early and specific carcinogenic factor in the development of skin cancers in humans (1, 5, 12, 25, 27, 35, 52, 54, 65, 66). According to the available literature, the sun emits three types of UVR: UVRA (320–400 nm), UVRB (280–320 nm) and UVRC (100–280 nm), which each damage DNA in a different way. In the skin carcinogenesis induced by UVRB, the formation of photoproducts such as cyclobutane pyrimidine dimers and pyrimidine (6-4)-pyrimidone leads to highly specific mutations arising, mainly single C → T and G → A and tandem CC → TT and GG → AA transitions, known as the "genetic fingerprint of UVR" (5, 10, 25, 52). The result of harmful UVRA exposure is the formation of reactive oxygen species (ROS), which leads to the appearance of several transitions similar to those caused by UVRB (C → T and G → A) and less commonly to other mutations, such as the T → C transition and the T → G and C → A transversions. The third type of radiation, UVRC, is highly mutagenic, but does not reach the Earth's surface, because it is almost completely blocked by the stratospheric ozone layer (52). Scientific literature widely describes UVR as one of the factors responsible for the development of squamous cell carcinomas (1, 5, 27, 65), basal cell carcinomas (5, 27, 65), malignant melanomas, and according to some authors, sweat gland carcinomas in humans (6). In dogs, exposure to UV light can cause squamous cell carcinoma, melanoma, haemangioma and haemangiosarcoma (56), but this statement is based more on clinico-epidemiological and immunohistochemical studies than molecular investigations (13, 55, 61). To the best of our knowledge, among the reports of *p53* mutations in dogs published so far (20, 24, 37–39, 44), none confirms UVR influence on canine skin carcinogenesis.

The main purpose of the present study was to investigate whether the *p53* gene is involved in the process of ASGC carcinogenesis in dogs, and whether the potential *p53* mutations in these cancers could be related to the influence of UVR.

Material and Methods

Material. The material for the study consisted of 40 archival paraffin blocks of canine ASGC tissues collected from national veterinary pathology laboratories in Poland between 2009 and 2014. The ASGCs accounted for 0.92% of all canine skin tumours diagnosed in these laboratories during that time.

Laser capture microdissection. For molecular analysis, the paraffin blocks were cut into 4 µm sections and stained with haematoxylin and eosin without coverslips. In order to visualise and select neoplastic cells under a microscope, a drop of xylene was applied to individual sections subjected to microdissection (Fig. 1). The laser capture microdissection was performed using an Arcturus XT system (Life Technologies, Carlsbad, CA, USA). The selected tumour cells were captured using a near-infrared laser pulse and transferred onto a macro cap (CapSure® Macro LCM, cat. no. LCM02; Life Technologies). DNA was extracted from the cells on the cap after overnight incubation with proteinase K extraction buffer at 56°C, using the Arcturus® PicoPure® DNA Extraction Kit (cat. no. KIT0103; Life Technologies) and 0.5 ml of autoclaved GeneAmp thin-walled reaction tubes (cat. no. N8010611; Applied Biosystems, Foster City, CA, USA). Following proteinase K digestion, the buffer was incubated at 95°C for 10 min and DNA was isolated and eluted in 20 µL of the buffer following the kit manufacturer's instructions.

PCR amplification and sequencing. For PCR amplification, samples of DNA extracted from the microdissected neoplastic tissues and the control DNA extracted from blood leucocytes of three healthy dogs with confirmed wild-type sequences of each exon of *p53* were used.

Oligonucleotide primers used in the PCR targeted conservative regions of canine *p53* (exons 5, 6, 7, 8) and the exons outside of these regions (exons 2, 3, 4, 9, 10, 11). The primer sets were designed for each exon separately according to published literature (8). The reaction mixture at a total volume of 25 µL contained 50 ng of DNA, 2.5 µL of polymerase buffer (10x Taq buffer: 100 µM, Tris-HCl, pH 8.8; 500 µM KCl; Fermentas, Vilnius, Lithuania), 2.0 mM $MgCl_2$ (25 mM $MgCl_2$ stock; Fermentas), 400 µM dNTPs (10 mM dNTP mix stock; Fermentas), 5 µM of each primer and 3 units of Taq polymerase (Taq polymerase 1 unit/µL stock; Fermentas). The PCR amplification conditions were as follows: initial denaturation at 94°C for 10 min followed by 45 cycles of 30 s denaturation at 94°C, 60 s primer hybridisation at 58°C and 60 s elongation at 72°C. Amplification was completed with elongation at 72°C for 5 min. The temperature profile and the composition of the reaction mixture were identical for all exons (2–11) of the *p53* gene.

Unincorporated primers and dNTPs were removed from the PCR products of individual exons of the *p53* gene and the products intended for sequencing were purified using ExoSap-IT (USB Affymetrix, Santa Clara, CA, USA) or Exo-BAP (EURx, Gdańsk, Poland) kits, which called for incubation in a Veriti 96-Well FAST Thermal Cycler (Applied Biosystems, Foster City, CA, USA) at 37°C for 15 min and then at 80°C for 15 min. To improve the sequencing quality and accuracy additional purification was performed using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the kit manufacturer's instructions. Due to the length of the PCR product of individual exons being between 74 bp (exon 3) and 204 bp (exon 4), 1–3 ng of the template was used for sequencing. Two separate cycles of sequencing reactions were carried out (one forward and one reverse) for each of the 11 exon specific products using a Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems).

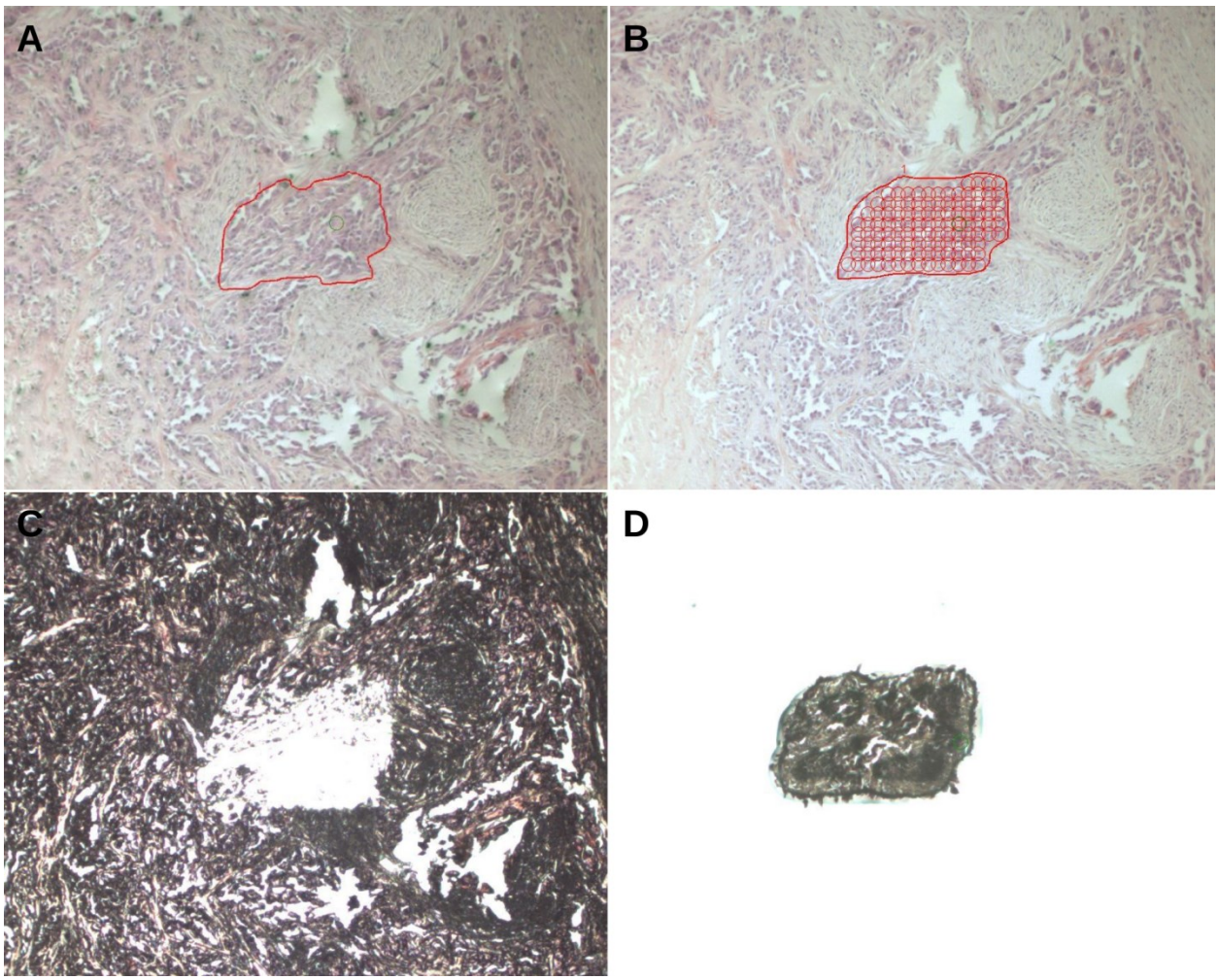

Fig. 1. Example images of the laser capture microdissection of tumour cells acquired using Arcturus XT operating software (10× objective). A – Haematoxylin and eosin stained section, covered with xylene for visualisation, showing cystic/papillary type apocrine sweat gland carcinoma before microdissection, with the area containing tumour cells marked with a red line. B – The same section with marked tumour cells and laser infrared spots (IR spots). C – The same section after xylene evaporation and the microdissection of the marked cells. D – Tumour cells transferred to the macro cap after laser capture microdissection

Sequencing reaction conditions were as follows: initial denaturation at 96°C for 60 s followed by 25 cycles of denaturation at 96°C for 10 s, primer hybridisation at 50°C for 5 s and elongation at 60°C for 4 min using the Veriti 96-Well FAST Thermal Cycler. The pre-sequencing products were purified using a BigDye X Terminator Purification Kit (Applied Biosystems) and then capillary electrophoresis was performed using an 8-capillary Hitachi 3500 Genetic Analyser sequencer (Applied Biosystems). The obtained sequences were trimmed and analysed using Geneious Pro 5.3 software (Biomatters, Auckland, New Zealand). To exclude Taq polymerase errors, a sequence analysis was performed twice for each of the exons of the *p53* gene. The consensus sequences corresponding to each amplified fragment were generated from each sample and compared with the sequence of canine control samples of *p53* (K1) and with the canine and human sequences of *p53* from GenBank at the National Center for Biotechnology Information (GenBank canine sequence accession numbers U62133 and AB020761 and human sequence accession number K03199). Sequences were aligned using the Geneious alignment module.

Results

The study yielded 16 *p53* mutations to detection confirmed in 13 ASGC samples, which constituted 31.7% of all the examined ASGCs (Table 1). Sequence analysis of the remaining 27 samples revealed mutation less *p53* genes. Among the 16 mutations, 11 missense point mutations, 1 tandem mutation, 3 silent point mutations and 1 single deletion were identified. The detected mutations were located both within the highly conserved region of the *p53* gene (exons 5–8) and outside it (exon 4). The majority of the identified mutations consisted of C →T, T → C and G → A single transitions. There was also one CC → TT double transition, one C → G transversion and one adenine deletion (Table 1). Six out of seven samples with mutations in exon 4 showed a specific substitution of phenylalanine for serine at position 90 of the *p53* protein

(Fig. 2), while three out of five samples with mutations in exon 5 showed substitution of alanine for valine at position 157 of the protein (Fig. 3). Mutations of *p53* were identified mainly in solid ASGCs, less frequently in ductal ASGCs and papillary ASGCs, and in single incidences in cystic/papillary and tubular ASGCs and as a carcinosarcoma (Table 1). Tumours in which the mutations of the *p53* gene were found were mainly located in the chest and forelimb. The age of the dogs with *p53*-mutated ASGCs ranged from 5 to 12 years with an average of 8.41 years, and mixed-breed dogs were most affected.

Table 1. Mutations in the *p53* gene in canine ASGC

Sample no.	Histology*	Breed	Age (years)	Body location	Exon	Codon †	Mutation (amino acid)
180	Papillary ASGC	—	5	Chest (rib area)	5	157	gTc → gCc (Val → Ala)
188	Papillary ASGC	German shepherd	6	Forelimb (arm area)	8	264	aAc →a-c reading frame shift
190	Solid ASGC	Mixed breed	8	Neck (side neck area)	5	178	caC → caT (His = His)
191	Ductal ASGC	Mixed breed	10	Chest (scapular area)	5	149, 150	tcCCca → tcTTca (Ser = Ser; Pro > Ser)
193	Solid ASGC	Dachshund	9	Forelimb (axillary area)	5	157	gTc → gCc (Val → Ala)
194	Tubular ASGC	Giant schnauzer	—	Finger	4 6	63 123 214	gcT → gcC (Ala = Ala) aCt → aGt (Thr → Ser) aCt → aGt (Thr → Ser)
201	Ductal ASGC	Mixed breed	12	Head (auricle area)	5	157	gTc → gCc (Val → Ala)
212	Papillary ASGC	Mixed breed	11	—	4	90	tCc → tTc (Ser → Phe)
217	Ductal ASGC	Mixed breed	5	—	4	90	tCc → tTc (Ser → Phe)
219	Cystic, papillary ASGC	Mixed breed	5	Pelvis (gluteal area)	4	90	tCc → tTc (Ser → Phe)
220	Solid ASGC	—	11	Pelvis (anal area)	4	90	tCc → tTc (Ser → Phe)
221	Solid ASGC	French bulldog	11	—	4	90	tCc → tTc (Ser → Phe)
222	Carcinosarcoma ASG	Miniature schnauzer	8	Chest (rib area)	4	90	tCc → tTc (Ser → Phe)

*ASGC – apocrine sweat gland carcinoma; ASG – apocrine sweat gland; † corresponding to human *p53* gene

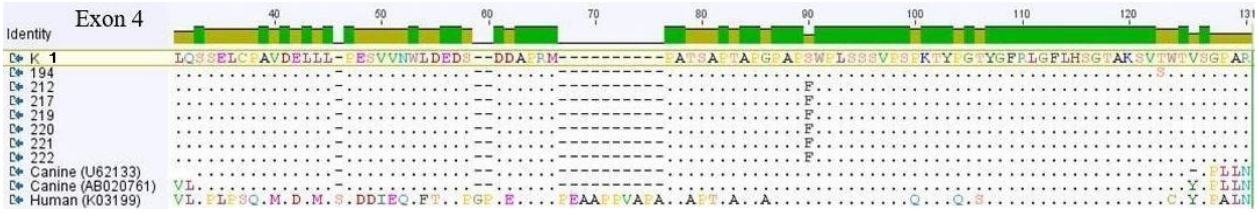

Fig. 2. Alignment of the amino acid sequence of exon 4 of the *p53* protein of the canine control (K1) sample and tested samples of apocrine sweat gland carcinomas with canine (U62133, AB020761) and human (K03199) amino acid reference sequences of the *p53* protein

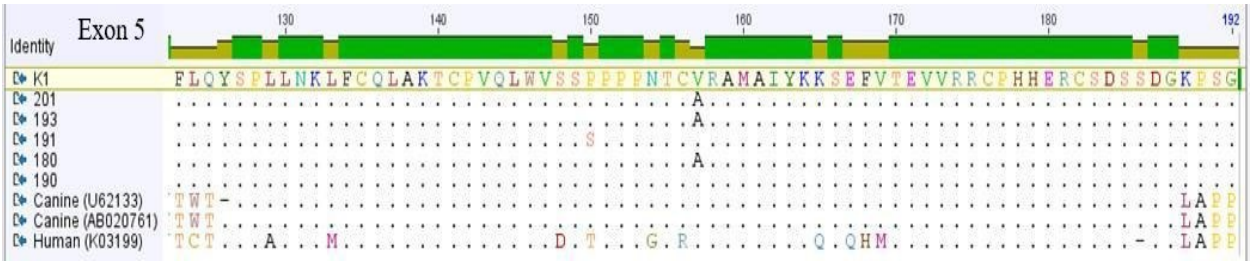

Fig. 3. Alignment of the amino acid sequence of exon 5 of the *p53* protein of the canine control (K1) sample and tested samples of apocrine sweat gland carcinomas with canine (U62133, AB020761) and human (K03199) amino acid references sequence of the *p53* protein

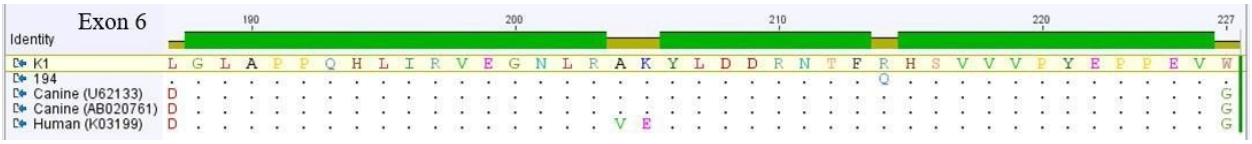

Fig. 4. Alignment of the amino acid sequence of exon 6 of the *p53* protein of the canine control (K1) sample and tested samples of apocrine sweat gland carcinoma with canine (U62133, AB020761) and human (K03199) amino acid reference sequences of the *p53* protein

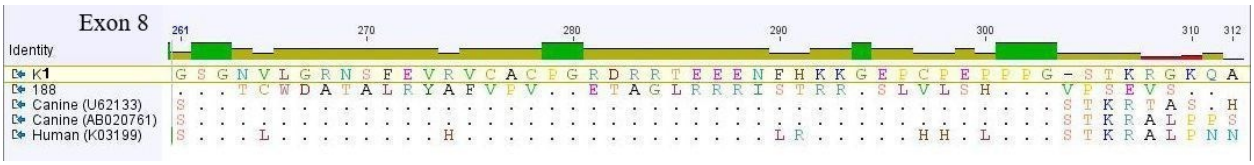

Fig. 5. Alignment of the amino acid sequence of exon 8 of *p53* of the canine control (K1) sample and tested samples of apocrine sweat gland carcinomas with canine (U62133, AB020761) and human (K03199) amino acid reference sequences of the *p53* protein

Discussion

Research on ASGCs published so far has not clearly defined which mechanisms are responsible for the formation of these tumours, although several publications are available describing mutations of the *p53* gene in single cases of ASGCs in humans (6) and dogs (20) and one report exists on the loss of heterozygosity confined to the 17p chromosome arm in humans (58). In the present study, 13 out of 40 canine ASGCs displayed *p53* gene alterations such as single C → T and T → C transitions and CC → TT double transition, which, according to previous reports, are characteristic of and specific to the involvement of UVR in skin carcinogenesis. The lack of *p53* mutations in the remaining cases suggests that this gene was not involved in the carcinogenesis of these tumours; putatively, these neoplasms are associated with other genes such as *H-ras* or *K-ras* (6).

The most prominent finding in our study was the detection of the TCC → TTC missense mutation at codon 90 changing serine to phenylalanine. It was common to 6 out of 13 ASGCs displaying *p53* mutations. Although this was the highest number of cases sharing the same *p53* mutation, there was not enough data available to determine any association of the mutation to breed, age, sex, tumour location or histological type. We failed to find any reports in the available literature on this mutation in any type of tumour. The GCT → GCC silent mutation found in another case at codon 63 has also not been previously reported to the best of our knowledge; however, there were other mutations at this codon revealed in human cancer, such as cytosine deletion considered to be a marker in oral squamous cell carcinoma (32, 40, 57) and the missense mutation changing alanine to proline in synovial sarcoma (9). Both codons 63 and 90 are located within a signalling proline-rich domain of the p53 protein between amino acids 61 and 94. The high proline content in this domain, especially sequence PXXP motifs (where P represents proline and X any amino acid), plays a critical role in the transmission of antiproliferative signals in response to DNA-damaging factors, leading to apoptosis (21).

At codon 157 in three ASGCs in our study, the missense mutation GTC → GCC changing valine to alanine was detected, which was consistent with the *p53* mutation described in canine osteosarcoma (26). This codon is located in the *β-sandwich* strand S4 of the DNA-binding core domain at residues 94–321, *p53*C, which plays a key role in the folding and stability of this domain of the *p53* gene (21) and is often mutated in colorectal, breast, liver, lung and ovary cancers in humans (30, 31). Single silent mutations associated with UVR at *p53* codon 157 were described in the stratum granulosum of squamous cell carcinoma (1) and in sporadic basal cell carcinoma cases (33) in humans. Studies on colorectal tumours published by Takata *et al.* (58) revealed single missense mutations and deletion/ insertion of several nucleotides, which resulted in a frameshift and truncated *p53* protein (59). Interestingly, the most common mutation at codon 157 documented by other authors is a valine to phenylalanine transition, which represents 77% of all mutations detected in this codon. This type of mutation located within the *β-sandwich* region causes global effects on the DNA-binding core domain and loop-sheet-helix motif, reducing the thermodynamic stability of the *p53* protein and causing

its unfolding at body temperature both *in vitro* and *in vivo*, and is classified as a globally denatured mutant (7, 47). Codon 157 is also considered to be a hotspot region associated with hepatoblastoma in children (45), a marker of poor prognosis in patients with hepatocellular carcinoma, and one of the most frequently mutated hotspot codons in smoking lung cancer patients (15, 51, 62, 64). Additionally, it has been found that cells containing alterations at codon 157 show medium radiosensitivity (46).

The same silent mutation at codon 178 (CAC → CAT) that was detected in one case in our study occurred in single cases of human sporadic basal cell carcinoma, nevoid basal cell carcinoma syndrome (Gorlin–Goltz syndrome) (33) and testicular carcinoma *in situ* (28). Several other mutations at codon 178 were detected in basal cell carcinoma (changing histidine to asparagine) (65) and in radiation dermatitis where the C → A transition led to the substitution of glutamine for histidine (27). Moreover, this codon falls in the hotspot regions in basal cell carcinomas in patients with the inherited disease of deficient DNA repair xerodema pigmentosum (12).

The other *p53* gene alterations revealed in the present study included the ACT → AGT missense mutation at codon 123, the CC → TT tandem mutation at codons 149–150 and the AAC → A–C deletion at codon 264 causing Asn to Tyr frameshift changing. To the best of our knowledge, none of these mutations have previously been described in any tumour. The only published studies on *p53* codon 123 were performed on the *Saos-2* human osteosarcoma cell line and revealed that cells with the threonine to alanine change at this location became highly radiosensitive (46). With regard to the alteration at codons 149–150, a TCC → TCT silent mutation was revealed at codon 149 in our study, in contrast to the findings of Hsu *et al.* (18). Those researchers detected a missense mutation at this gene position changing serine to phenylalanine in patients with precancerous Bowen's disease caused by UVR and chronic exposure to a high level of arsenic contamination in drinking water (18). Additionally, another missense mutation at codon 149 (TCC → CCC) changing serine to proline was documented in oral squamous cell carcinomas (43), whereas in a report on colorectal cancer by Tang *et al.* (59) there was an insertion of thymine at this location of the *p53* gene, generating a frameshift that resulted in a truncated *p53* protein. At codon 150 there was a missense mutation identified in the present study, which has not been detected in any species so far. According to some authors, UVRA penetrates the skin deeper than UVRB (49, 52), which suggests that UVRA might be responsible for carcinogenesis in canine sweat glands due to their location in the skin. However, the types of mutation described in our study appear to be associated with the effect of both UVRB (C → T, G → A and CC → TT) and UVRA (T → C, C → G and single deletions) accounting for 60% and 40% of all mutations detected, respectively.

In conclusion, 31.7 % of the examined canine ASGCs have been confirmed to be affected by *p53* mutations, the majority of which, including the mutation detected in six cases in exon 4, have never been reported in any tumour. Moreover, the characteristics of all the detected mutations are consistent with those of UVR-induced gene alterations. Our results may therefore indicate the involvement of both the *p53* gene and UVA and UVB radiation in the formation of ASGCs in dogs. Moreover, these results revealed new and important mutations associated with the function of the *p53* gene.

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