SHORT COMMUNICATION

Mutation of the *p53* tumor suppressor gene in spontaneously occurring osteosarcomas of the dog

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Inactivation of the *p53* tumor suppressor gene has been implicated in the pathogenesis of numerous human cancers, including osteosarcomas. Appendicular osteosarcomas of the dog appear to be a good model for their human equivalent with regard to biologic behavior, epidemiology and histopathology. We individually screened exons 5–8 of the *p53* gene for mutations in 15 canine appendicular osteosarcomas using ‘Cold’ SSCP to compare the role of this gene in human and canine osteosarcoma tumorigenesis. Seven of the tumors (47%) exhibited point mutations, with one tumor possessing two mutations within different exons. Of these, seven were missense mutations and the eighth was a ‘silent’ mutation potentially affecting the exon 6–7 splicing region. Five of the missense mutations were located in highly conserved regions IV and V, while another corresponded with the highly conserved codon 220 mutational hotspot located outside the conserved domains. The locations and types of mutations were nearly identical to those reported in human cancer. These findings provide strong evidence of the involvement of *p53* mutations in the development of canine appendicular osteosarcomas. Canine osteosarcomas appear to be a promising model for their human equivalent on a clinical, pathologic, and molecular level.

The pathogenesis of a wide variety of human neoplasms includes mutational inactivation of the *p53* tumor suppressor gene (1,2). The encoded nuclear phosphoprotein serves as a negative regulator of cellular proliferation, promotes DNA repair, and plays a role in apoptosis (3–5). Alterations of *p53* protein function may allow unrestricted cellular proliferation and subsequent development of tumors. Deletions, translocations or mutations of the *p53* gene are observed in ~60% of human cancers (5). In most tumors, both *p53* alleles are inactivated, one by a mutation and the other by deletion. Exons 5–8 of the *p53* gene contain up to 98% of *p53* mutations observed in human tumors and contain four of five evolutionarily conserved regions, which appear critical to normal function (2). The presence of *p53* mutations is associated with aggressive biologic behavior, poor prognosis and altered response to therapy in many human tumors (1.6–8).

Human osteosarcomas (OSAs*) also have a high prevalence of *p53* gene alterations. Allelic deletion of *p53* has been demonstrated in over 70% of human OSAs (9–13), while point mutations, small deletions, insertions and splice mutations are identified in 18–100% (13–17). Gross rearrangements or partial deletions of the *p53* gene were demonstrated in an additional 24–50% of human OSAs (11,13,18). Immunohistochemistry is also supportive of a high prevalence (23–72%) of *p53* alterations in human and canine OSAs (19–23). Osteosarcoma is also one of the sarcomas occurring in excess in families possessing germ-line mutations of the *p53* gene, and is often used to define probands for epidemiologic studies of Li-Fraumeni syndrome (14,24–28).

Several studies further demonstrate the important role of the *p53* gene in the development of OSA, as a determinant of clinical behavior and therapeutic response, and as a potential basis for development of future therapy. Transgenic mice with a mutant or inactivated *p53* gene have a high frequency of OSAs (29,30). Reintroduction of the wild-type *p53* gene into *p53*-deficient metastatic human OSA cell lines results in terminal differentiation and apoptosis in vivo, and suppression of growth in vitro (7,31,32). Finally, reintroduction of a normal *p53* gene and subsequent expression of a normal *p53* protein product resulted in increased radiosensitivity of *p53*-deficient cell lines, while expression of abnormal *p53* protein decreased radiosensitivity (33). Similar effects may be seen regarding susceptibility to chemotherapy and radiation therapy in human tumors (6,8).

Since 1978, the dog has been proposed as a model for studies of human cancers (34). The most frequently compared canine cancer is OSA (35–37). OSA is the most common primary bone tumor in both humans and dogs (38,39). There are striking similarities between OSAs in the dog and humans regarding gender predilection, relative body mass, anatomic site, histologic grade and subtypes, high metastatic rate and prognosis (40,41). Canine OSA has a later onset, earlier onset of metastasis, and ~40 times the prevalence of the human equivalent (39,41). It is estimated that over 8000 new cases of spontaneous canine OSA occur annually in the United States (41). This may represent a significant underestimate, since many cases of canine OSA are never diagnosed. In addition, tumors in dogs and humans often have a similar response to radiation and chemotherapy (3,34,36,42).

Given these similarities and the high frequency of canine OSAs relative to humans, dogs are potentially an ideal model for examining the role of *p53* in the development and biological behavior of this neoplasm. Canine appendicular OSAs, in particular, are an attractive model due to their relative frequency and aggressive behavior. These tumors account for 75–80% of OSAs in the dog and develop pulmonary metastases earlier and more frequently than those of the axial skeleton, often before a primary tumor is identified (40,43). As a result, appendicular OSAs represent one of the most biologically aggressive tumors encountered in the dog. This study attempts to define the role of *p53* gene alterations in the development of canine appendicular OSAs for the purpose of identifying

*Abbreviations: OSAs, osteosarcomas; H&E, hematoxylin and eosin; PCR, polymerase chain reaction; SSCP, single-stranded conformation polymorphism; LOH, loss of heterozygosity.*
similar or dissimilar molecular mechanisms of tumor development compared with their human counterparts.

Formalin-fixed, paraffin-embedded surgical biopsy submissions of 15 spontaneous appendicular canine OSAs were obtained from the Ohio State University Veterinary Pathology Library. Serial 8-µm sections were cut and mounted individually on slides. Disposable microtome blades were utilized and changed following sectioning of each sample to prevent cross-contamination of DNA. One slide from each tumor was stained with hematoxylin and eosin (H&E) to confirm the presence of neoplastic tissue and assist in microdissection of tumor tissue from unstained slides. To gain access to microscopically discrete areas of tumors, affected areas were identified and microdissected from paraffin-embedded tissue sections. Selected areas were carefully scraped from single, unstained sections into a tube using a sterile disposable surgical blade and the corresponding H&E stained section as a visual template. Selected areas were carefully scraped from single, unstained sections into a tube using a sterile disposable surgical blade and the corresponding H&E stained section as a visual template. Octane (500 µl) was added to each of the scraped samples to deparaffinize the tissue. Tubes were gently vortexed for 20 min and then centrifuged for 6 min at 14,000 g using a microcentrifuge. The octane was removed, and the pellet was washed with 500 µl of 100% ethanol for 20 min and then air-dried for 2 h. The dried pellet was resuspended in 100 µl of digestion buffer (50 mM Tris, pH 8.5, 1 mM EDTA, and 0.5% Tween 20) containing 200 ng/ml of proteinase K and incubated at 55°C for a minimum of 24 h. The suspension was then microcentrifuged for 10 s, heated to 95°C for 10 min to inactivate the proteinase, and finally clarified in a microcentrifuge tube for 1 min. The supernatant was stored at 4°C and used as the DNA template in subsequent polymerase chain reaction (PCR) amplifications.

In order to fully evaluate all of the nucleotides associated with exons 5–8, as well as intron/exon splice junctions, PCR primers must be designed from intron sequences. This strategy also serves to avoid co-amplification of potential p53 pseudogenes that have been identified in other species (44,45). Therefore, canine p53 introns 4, 7 and 8 were amplified using primers obtained from published canine p53 exon sequences (Table I) (46). PCR was performed using 30 cycles of denaturation (94°C, 50 s), annealing [50 s at the appropriate T(a); see Table I], and extension (72°C, 50 s). PCR products were purified and sequenced as described below. From the intron sequences thus obtained (GenBank accession numbers AF024718, AF024719 and AF024720) and previously published sequences of introns 5 and 6 (47), four pairs of intron-based PCR primers were designed for amplification of p53 exons 5–8 and their flanking intron regions (Table I). PCR amplification of exons 5–8 was performed in duplicate for each tumor sample to allow confirmation of detected mutations and identification of potential random Taq-induced mutations. Hot-start PCR utilizing Taq antibody was performed to avoid priming of non-specific templates (48,49). PCR was performed using 35–40 cycles of denaturation for 30 s (94°C), annealing for 30 s at the appropriate T(a) (see Table I), and extension for 30 s (72°C), followed by a final 7-min extension at 72°C. For each set of PCR reactions, a negative control containing no template was employed. PCR products were analyzed using 10% polyacrylamide mini-gels (Novex, San Diego, CA) at 250 V for 30 min, stained with ethidium bromide (0.5 µg/ml in 1× TBE buffer) for 10 min, and visualized by UV fluorescence.

PCR fragments generated from amplification of p53 exons 5–8 were analyzed by single-stranded conformation polymorphism (SSCP). The method of ‘Cold’ SSCP was followed as described previously (50). A mixture consisting of 1–5 µl of PCR product (20–200 ng of DNA), 0.4 µl of 1 mM methylmercury hydroxide, 1 µl loading buffer (15% Ficoll, 40,000 MW, 0.25% bromophenol blue, 0.25% xylene cyanol), and 13.6 µl of 1× TBE buffer was prepared for each PCR product to yield a total volume of 16–20 µl. This mixture was heated to 85°C to denature double-stranded DNA, then plunged into ice prior to loading the entire volume on the gel. Precast, 8×8 cm, 0.1 cm thick, and 20% polyacrylamide TBE mini-gels were used with the Novex Thermoflow SSCP System (Novex, San Diego, CA). The buffer chamber was filled with 1.25× TBE buffer. A constant (±0.5°C) buffer temperature was maintained within the cell chambers during the gel run. Positive control PCR products for each exon were generated by the ‘megaprimer’ method of site-directed mutagenesis (51) using primers described in Table I. Optimal buffer temperatures of 1°C, 9°C, 7°C and 15°C were empirically determined for the p53 exon 5–8 fragments, respectively, to attain maximal differentiation between wild-type and positive control samples. Gels were run at 300 V (38 V/cm) for 3–10 h, depending on the fragment size and buffer temperature, then stained with SyberGreen (Molecular Probes, Eugene, OR) in TBE buffer for 10 min at room temperature and photographed under UV fluorescence. Evaluation of products from duplicate PCR reactions was employed for each sample to confirm positive shifts in SSCP bands.

Mutant bands were isolated from the SSCP gel and re-amplified 20 cycles by PCR to enrich for mutant alleles. Following PCR, enriched mutant products were purified and concentrated into 40 µl using Micron-100 microconcentrators (Amicon, Beverly, MA) to remove salts, primers and dNTPs. Purified PCR mutant products were sequenced on an Applied Biosystem 377 Prism DNA Sequencer using an ABI PRISM™ dye terminator cycle sequencing ready reaction kit (ABI/PE, Foster City, CA) according to the manufacturer’s instructions. Electrophoresis was performed at 3000 V for 3.5 h and DNA sequences were analyzed using ABI sequencing analysis software. All mutations were confirmed by sequencing both DNA strands. Exons amplified from normal tissues, corresponding to tumor tissues containing mutations, were also sequenced to identify possible germ-line defects and to eliminate the possibility of pseudogene amplification or breed-associated polymorphisms.

Silent somatic mutations within coding portions of the p53 gene were analyzed for potential effects on mRNA splicing using the Signal subprogram within the PC/Genie (Intelligenetics, Mountainview, CA) software package. Exons 5–8 of the p53 gene were screened individually for mutations in 15 canine appendicular OSAs. Breeds evaluated were: Labrador Retriever (3), Rottweiler (2), Doberman Pinscher (2), mixed breed (2), old English Sheepdog, German Shepherd Dog, Irish Setter, Great Pyrenees, Great Dane and Gordon Setter. Mutation associated SSCP band shifts were identified and confirmed in seven tumor samples (47%), one of which possessed mutations in both exons 6 and 7. An example SSCP analysis identifying a mutation is shown in Figure 1. Sequence analysis identified seven missense point mutations and a silent point mutation located at the junction of exons 6 and 7. These are summarized in Table II. Three of the mutations (codons 188, 220 and 224 of the human gene) occurred outside of the five highly conserved domains. The remaining mutations fell within highly conserved domains IV and V. Two identical mutations were observed in independent
Table I. Primers used for sequencing and SSCP analysis of the canine p53 gene

<table>
<thead>
<tr>
<th>Product location</th>
<th>Product size (bp)</th>
<th>Primer sequence (3' to 5')</th>
<th>T(a) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 4</td>
<td>707</td>
<td>GACCTACCCTGGCAGCTATGG (+)</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TACGAAACTCCAGCTCTTATA (-)</td>
<td></td>
</tr>
<tr>
<td>Intron 7</td>
<td>329</td>
<td>ACTATCATTACCCCTGAAGAC (+)</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCACAAAAGGGTACCTCAAAAG (-)</td>
<td></td>
</tr>
<tr>
<td>Intron 8</td>
<td>~375</td>
<td>AGACCGGCGACTGAGGAGGA (+)</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACGTGGTAGGGGAAGGCAATGG (-)</td>
<td></td>
</tr>
<tr>
<td>Exon 5</td>
<td>249</td>
<td>AACGTGTCAATCCTGCTTTTC (+)</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGCTTGTCCTACAATCTCAGG (-)</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>204</td>
<td>ATGCCTCCGGATGCTCTTA (+)</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTTTTTTCTTACTAAGTTAACCCC (m)</td>
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<td>GGCACCTGGGCTACCTCT (+)</td>
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<td></td>
<td></td>
<td>GGCACCTGGGCTACCTCTCACCTGTCGTC (m)</td>
<td></td>
</tr>
<tr>
<td>Exon 8</td>
<td>193</td>
<td>CGGGACCTGGGCTACCTCGT (+)</td>
<td>64</td>
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<tr>
<td></td>
<td></td>
<td>CCTCACCTTCTCTTTTGCTTG (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGGGACCTGGGCTACCTCTCACCTGTCGTC (m)</td>
<td></td>
</tr>
</tbody>
</table>

a (+) sense primer, (–) antisense primer, (m) primer with induced mutation (underlined) for positive control.

Table II. Point mutations identified in osteosarcoma tumor tissues

<table>
<thead>
<tr>
<th>Tumor sample</th>
<th>Breed</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
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<tbody>
<tr>
<td>OSA-1</td>
<td>Great Pyrenees</td>
<td>6</td>
<td>224</td>
<td>GAG→GAA</td>
<td>noneb</td>
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<tr>
<td>OSA-2</td>
<td>mix</td>
<td>7</td>
<td>249</td>
<td>CGG→TGG</td>
<td>Arg→Trip</td>
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<tr>
<td>OSA-10</td>
<td>Doberman Pinscher</td>
<td>7</td>
<td>249</td>
<td>CGG→TGG</td>
<td>Arg→Trip</td>
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<tr>
<td>OSA-19</td>
<td>Irish Setter</td>
<td>6</td>
<td>220</td>
<td>TAT→TCT</td>
<td>Tyr→Ser</td>
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<tr>
<td>OSA-23</td>
<td>Rottweiler</td>
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<td>258</td>
<td>GAA→AAA</td>
<td>Glu→Lys</td>
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<tr>
<td>OSA-25</td>
<td>mix</td>
<td>6</td>
<td>188</td>
<td>TT→ATT</td>
<td>Leu→Ile</td>
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<tr>
<td>OSA-29c</td>
<td>Labrador Retriever</td>
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<td>276</td>
<td>GCC→GAC</td>
<td>Ala→Asp</td>
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<tr>
<td>OSA-29d</td>
<td></td>
<td>7</td>
<td>248</td>
<td>CGG→CAG</td>
<td>Arg→Gln</td>
</tr>
</tbody>
</table>

a Corresponding to human p53 gene.
b Silent mutation located at the junction of exons 6 and 7, resulting in reduced affinity of the cryptic donor splice site.
c An identical mutation was identified in a metastasis from the same animal.

g Fig. 1. ‘Cold’ SSCP analysis of canine p53 exon 8. Positive SSCP band shifts are present (arrows) in duplicate PCR reactions (lanes 29A and 29B) from a single osteosarcoma (OSA-29) which are different in mobility compared with the corresponding wild-type bands (WT). Note that a majority of the alleles comprise the mutant band, while only a small proportion of the wild-type alleles remain. Enrichment of the mutant fragment was accomplished by removing a plug from the shifted band in the original SSCP gel followed by reamplification, as seen in lane 29E. The band shift was later attributed to a missense substitution. A positive control fragment created by site-directed mutagenesis also demonstrated an appropriately unique band shift (PC).

tumor samples at human codon 249 in the fifth highly conserved domain. Five of the eight mutations observed were G:C→A:T transitions, two were G:C→T:A transversions, and one was an A:T→C:G transversion. Three of the G:C→A:T transitions occurred at CpG sites.

Mutations were not identified in corresponding normal tissues from six of the seven cases where normal tissues were available for evaluation. Normal tissue from one case (sample OSA-19) was not available. However, the SSCP profile from this sample demonstrated the shifted mutant band to be comprised of the majority of amplified alleles (85–90%) with a faint wild-type band also being present. Thus, the nucleotide change responsible for the SSCP band shift represents a legitimate mutation, since a breed-associated polymorphism would have resulted in a mutant band shift containing either 50% or 100% of an altered allele.

We have demonstrated that p53 mutations are present in canine OSAs at a frequency comparable to or greater than that of human OSAs. Of the seven missense mutations observed, five are located in the five highly conserved domains and one is at the junction of exons 5 and 6. The remaining missense mutation (codon 220) is located at one of two mutational hotspots in a small, highly conserved region at codons 213–223 (1). Finally, the silent mutation (codon 224) located at the exon 6–7 junction may be functionally relevant since it reduces the affinity of the appropriate cryptic donor splice site relative to other surrounding potential splice sites (data not shown). Thus, each mutation identified is likely to have an impact on p53 function. Since tumor-associated mutations were not identified in corresponding normal tissues, they do not represent
germline mutations, pseudogenes or breed-associated polymorphisms.

The p53 point mutations reported here in the dog correspond precisely to inactivating mutations previously identified in the human p53 gene in various cancer types, with the exception of codons 188 and 224 (2,53). Alternate missense point mutations have been reported at codon 224, while frame-shift mutations have been identified at codon 188, one of which was a human OSA (52). Three mutations correspond to human codons 248 and 249, which are sites of mutations in human OSAs (52,53) and represent two of five mutational hotspots accounting for 23% of reported p53 mutations in human cancer (1.54).

G:C→T:A transitions represent the majority (47%) of all p53 gene point mutations identified in human cancers and 62.5% of the mutations identified in this study (12). G:C→T:A transitions account for 25% of the mutations in our study and are the second most common p53 gene point mutation (20%) identified in human cancers. Of the five G:C→A:T transitions observed, 60% occurred at CpG sites, as compared with 24% in all human cancers. In general, G:C→A:T transitions at CpG sites represent endogenous DNA damage resulting from deamination of 5-methylcytosine (1,2). Over one-half of p53 germ-line mutations are of this type. G:C→T:A transitions and non-C→A:T transitions, on the other hand, are generally associated with carcinogen exposure. It should be noted that the human codon 249 is AGG versus CGG in the dog, resulting in CpG sites at the five most common sites of p53 mutation in human cancers (1,2,46).

While loss of heterozygosity (LOH) was not evaluated critically, mutant SSPC bands were substantially brighter than wild-type bands in 75% of the samples, suggesting that the wild-type p53 gene is not present in the majority of cells in most samples (data not shown). Minor amounts of wild-type p53 are expected as a result of contamination with normal cells or subpopulations of tumor cells not displaying LOH.

The p53 mutations reported here provide strong evidence of the role of this gene in the development of canine appendicular OSAs in a manner parallel to that demonstrated in human OSAs. Where p53 mutations were not observed, other mechanisms of p53 inactivation may exist. Mutations may be present in unexamined regions of the p53 gene or its promoter. Alternatively, overexpression of MDM2 in OSAs can bind and inactivate normal p53 protein (15,55).

The possible role of alternative gene products in the development of canine neoplasia should not be discounted. Germ-line and spontaneous RB1 gene alterations are associated with the development of human, and possibly canine, OSAs (10,12,15,16,56–58). One study reported possible RB deletions in 9 of 10 canine OSAs evaluated (57). The oncogenes Ki-ras and c-myc have been implicated in the development of other canine tumors (59,60). Nevertheless, this study provides strong evidence for the involvement of p53 alterations in the development of canine appendicular OSAs. These tumors appear to be a promising model for their human equivalent on a clinical, pathologic, and molecular level.

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References


