Mutational analysis of tumor suppressor gene p53 in feline vaccine site-associated sarcomas

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Objectives—To investigate the role of tumor suppressor gene p53 mutation in feline vaccine site-associated sarcoma (VSS) development and to evaluate the relationship between p53 nucleotide sequence and protein expression.

Sample Population—Formalin-fixed paraffin-embedded tissues of 8 feline VSS with dark p53 immunostaining (high p53 expression) and 13 feline VSS with faint or no staining (normal p53 expression).

Procedure—DNA was extracted from neoplastic and nonneoplastic tissues from each paraffin block. The following 3 regions of the p53 gene were amplified by polymerase chain reaction: 379 base pair (bp) region of exon 5, intron 5, and exon 6, 108 bp region of exon 7, and 140 bp region of exon 8. Amplified p53 products were sequenced and compared with published feline p53. The p53 mutations identified were correlated with p53 mutations predicted by immunostaining.

Results—Neoplastic cells of 5 of 8 (62.5%) VSS that had high p53 expression harbored single missense mutations within the p53 gene regions examined. The p53 gene mutations were not detected in the 13 tumors with normal p53 immunostaining. Nonneoplastic tissues adjacent to all 21 VSS lacked mutations of these p53 gene regions.

Conclusions—The p53 gene mutations were restricted to neoplastic tissue and, therefore, were unlikely to predispose to VSS. However, p53 mutations may have contributed to cancer progression in 5 of the 21 VSS. There was very good (κ = 0.67 with a confidence limit of 0.3 to 1.0) agreement between prediction of mutation by p53 immunostaining and identification of mutations by sequencing of key p53 gene regions. (Am J Vet Res 2000;61:1277–1281)

Sarcomas have been reported to develop at sites routinely used by veterinarians for vaccination in cats. The cause and pathogenesis of these tumors are unknown, and the roles of vaccination and chronic inflammation are currently under debate. The low prevalence of vaccine site-associated sarcomas (VSS), at 1 or 2/10,000 vaccines administered,1–4 suggests that affected cats belong to a subpopulation of susceptible cats. Susceptibility factors for VSS development have not been elucidated; however, mutation of a tumor suppressor gene such as p53 may play a role.

Mutations in the p53 gene are commonly implicated in cancer initiation and progression in humans.5–8 Inherited p53 mutations have been documented in carcinomas of breast and adrenal cortex, soft tissue sarcomas, leukemias, and brain tumors.7 Acquired p53 mutations resulting from endogenous and exogenous mutagenic mechanisms are seen in several types of human cancers.8,9

Tumor suppressor gene p53, a transcription factor involved in growth control, is critical for the cellular repair response to DNA-damaging agents and is an inducer of apoptosis, which aids in curtailing neoplastic growth.4 In an environment such as a vaccination site that may comprise inflammation, necrosis, and cell proliferation, a normal p53 gene product would be necessary for proper DNA repair and replication or to signal apoptosis in cells with irreparable DNA damage.

The p53 gene is well conserved across various species, comprises 11 exons and 10 introns, and encodes a protein of 393 amino acids. Exons 5 through 8 of the protein-coding region of p53 gene contain 5 conserved domains (1 to V). Most p53 gene mutations identified are within the conserved domains. Domains II to V of p53 protein are within the central DNA-binding region that is the most important functional component of the p53 protein.10,11 The feline p53 gene, which shares 82% homology with the human gene, has been completely sequenced and assigned to feline chromosome E1.12,13

The p53 gene has been investigated only minimally in animal tumors. Recently, mutations within the central region of the p53 gene were identified and their nucleotide sequence determined in 2 fibrosarcomas, 2 mammary carcinomas, 1 lymphosarcoma, and 1 osteosarcoma from cats, and 1 canine papilloma.14–16 Detection of p53 protein by immunohistochemistry (IHC) has been used as a surrogate for mutational analysis of p53 gene in many human tumors. Mutation within the p53 gene can stabilize the protein, thereby increasing its half-life and allowing detection by IHC,8,10,12 whereas the normal p53 gene product is below immunohistochemical detection limits. In a previous IHC study of 40 VSS,17 p53 protein expression was high in 42.5% of the tumors, whereas the remaining 57.5% had no or faint staining indistinguishable from unaffected tissues. The objectives of the study presented here were to determine p53 nucleotide sequence and protein expression in feline VSS.
sequences in neoplastic and nonneoplastic cells from 21 VSS, to evaluate the potential role of preexisting p53 gene mutations in VSS development, and to establish the relationship between p53 gene mutations and protein expression by IHC.

**Materials and Methods**

**Tissues**—Twenty-one VSS were chosen from a group of 40 that had been stained immunohistochemically with CM1 p53 antibody in a previous study. All tumors were formalin-fixed paraffin-embedded and identified as fibrosarcomas on histologic examination. Tissue blocks were obtained from 3 different diagnostic laboratories. Only tumors that were vaccine-associated on the basis of anatomic location, histologic features, and vaccination history (when available) were included. Tissues chosen for our study consisted of 8 tumors that stained darkly (equal to that of the COS 7 positive control, cells that lack endogenous p53) and 13 tumors that stained faintly or did not stain (equal to that of an unaffected canine epithelium) with the CM1 p53 antibody. In addition to staining patterns, tumors were chosen on the basis of ample adjacent nonneoplastic tissue (eg, epidermis and superficial dermis) and sufficient separation of neoplastic tissue and nonneoplastic tissue to avoid cross-contamination of these tissues during DNA extraction.

**DNA extraction**—Tissue retrieval was performed after examining the paraffin block in relation to the histologic section. Using standard techniques to prevent DNA carryover, 3 mm2 (0.1 to 0.15 mg) of neoplastic and nonneoplastic tissue were manually dissected from each block, using a scalpel blade and a dissection microscope. The DNA extraction was performed, using a commercial kit according to the manufacturer's instructions. The xylene paraffin extraction step was bypassed. The DNA extracted in parallel from formalin-fixed paraffin-embedded and identified as fibrosarcomas on histologic examination was purified by electrophoresis through low melting point agarose and subsequently eluted from the gel, using a commercial extraction kit as per the manufacturer's directions. The concentration of the amplified p53 gene regions was estimated by comparison with a commercially available DNA mass ladder after electrophoresis in a 2% agarose gel. Purified DNA samples representing the amplified regions were submitted to another laboratory for nucleotide sequencing in sense and antisense directions. Sequence data from the 21 VSS were compared with defined feline p53 gene sequence. The numbering of the feline p53 gene sequence was done in accordance with the human sequence to maintain uniformity. When mutations were identified, PCR and sequencing were repeated to confirm the nucleotide data.

**Statistical analysis**—The amount of agreement between sequence data and IHC for detection of mutations in the p53 gene was measured by calculating the kappa quotient. Kappa is defined as a measure of agreement beyond that which may be expected as a result of chance. A kappa of 0 indicates no agreement beyond chance, and a kappa of 1 indicates perfect agreement. Moderate agreement is indicated by a kappa of at least 0.4 to 0.5.

**Results**—Exons 5 through 8 and intron 5 of the feline p53 gene were sequenced successfully from all samples. Nonspecific bands were not observed on the agarose gels, and all the amplified products were intense, discrete bands of the appropriate bp size on AGE. Single missense mutations resulting in amino acid substitutions were found in the neoplastic tissue of 5 VSS. In all 5 mutations, the nucleotide chromatograph had 2 superimposed peaks representing expected and variant bases at the site. Mutations and codon positions for the 5 VSS are shown (Table 1). The neoplastic cells in these 5 VSS had dark nuclear staining by IHC. Agreement was very good (κ quotient = 0.67 with a confidence limit of 0.3 to 1.0), between p53 mutational analysis and protein expression (Table 2). Mutations in the p53 gene were not identified in nonneoplastic tissues of the 21 samples. However, a variation in the codon sequence at position 163, TAT and TAC, which encode the amino acid tyrosine, was identified in neoplastic and nonneoplastic tissues within the evaluated VSS. Two tissues contained the homozygous codon sequence TAT/TAT, 7 contained the
ated sarcomas


Table 2—p53 mutation detection by nucleotide sequencing and immunohistochemical staining in 21 feline vaccine site-associated sarcomas

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<thead>
<tr>
<th>Variable</th>
<th>Increased staining</th>
<th>Normal staining</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutations +</td>
<td>5</td>
<td>0</td>
<td>5/21</td>
</tr>
<tr>
<td>Mutations −</td>
<td>3</td>
<td>13</td>
<td>16/21</td>
</tr>
<tr>
<td>Total</td>
<td>8/21</td>
<td>13/21</td>
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Kappa quotient is 0.67, indicating very good agreement between sequence analysis and immunohistochemistry to detect p53 mutations. This value is significant (P < 0.05). The 95% confidence limits for this kappa value are 0.3 to 1.0.

tyrosine163, and within codons 193 to 195 and codons 213 to 220. Tyrosine163 sits adjacent to arginine248, 1 of the residues known to play an important role in maintaining the structural integrity of the DNA binding surface of the p53 protein. Therefore, tyrosine163 substitution by cysteine163 is likely to alter the structural integrity of the DNA binding surface of p53 protein.1

In our study, histidine214 and tyrosine234 were substituted by arginine and cysteine, respectively, and no data are available regarding the functional importance of these mutations.

At the nucleotide level, 3 adenine to guanine mutations, 1 guanine to thymine, and 1 cytosine to thymine were found. One of these 5 mutations (cytosine to thymine) occurred at a CpG dinucleotide within arginine248. The CpG dinucleotides are prone to methylation of the cytosine base to 5-methylcytosine that can then undergo spontaneous deamination to thymine. Thus, a missense mutation may occur as cystosine is replaced by thymine.24,26 Generally, CpG dinucleotide mutations often indicate spontaneous endogenous events rather than an exogenous influence.24 Methylcytosine is said to increase the potential for mutation at cytosine residues by a factor of at least 10.27

In all 5 mutations, the chromatograph that had the nucleotide sequence had 2 superimposed peaks representing expected and variant bases at the site. This may have been the result of PCR amplification of mutant and normal p53 from 2 different tumor cell types because of tumor heterogeneity. Genetic variability of p53 gene in neoplastic cells within the same tumor would suggest p53 mutation may occur as a late event in progression of VSS.

Alternatively, p53 allelic heterozygosity with mutant and nonmutant genomes may exist within the same cell. Confirmation would require single-cell microdissection followed by p53 gene amplification and nucleotide sequencing. The mutant allele may act in a dominant negative fashion by inactivating the normal (wild type) gene function.26,28 Mutant p53 protein may bind to wild type p53 resulting in heterotetramers that are not functionally efficient, compared with the homotetramers. This could result in a reduction of wild type p53 activity and less efficient suppression of growth.26

A third explanation for the 2 different bases at the same site may be contamination of tumor tissue with nonneoplastic tissue occurring during dissection of the samples. Extreme care was taken to extract DNA from representative tissues within the VSS, but contamination cannot be completely ruled out.

The nonneoplastic cells adjacent to the tumor tissue lacked p53 mutations within the regions examined.

Discussion

The p53 gene product is a component in biochemical pathways central to human carcinogenesis as loss of normal p53 function, usually caused by genetic mutation, provides an advantage for clonal expansion of preneoplastic and neoplastic cells.7 In our study, tumor cells from 5 of 21 (24%) VSS contained single missense mutations within exons 5 through 8 and intron 5 of p53. This mutation rate is similar to reports in sporadic human sarcomas that have an occurrence of 30%.24

The mutations involving codons 238 and 248 are located in the well-conserved domain IV (codons 236 through 258). The amino acid residues 236 to 251 form a loop that binds to DNA within its minor groove.25 Cysteine at codon 238 (cysteine238) acts as 1 of the 4 binding sites for the zinc atom in the p53 protein. This zinc atom is important in maintaining the structural integrity of the DNA-binding functional region of p53 protein. Cysteine238 mutation accounts for 1.8% of the mutations reported in p53 gene.1 In our study, substitution of hydrophilic cysteine238 by hydrophobic phenylalanine was likely to alter the conformation of the zinc binding site, resulting in loss of structural integrity of the central region and subsequent loss of p53 function.

Arginine at codon 248 (arginine248) contacts the minor groove of the DNA and is the most commonly mutated residue of p53 in human cancers (9.6% of p53 mutations).8 Missense mutation at codon 248, resulting in arginine substitution by tryptophan, is known to inactivate p53 function because of replacement by an amino acid of different charge and physical properties.

In our study, single missense mutations were also found at tyrosine163, histidine214, and tyrosine234 in 3 VSS. Although histidine214 and tyrosine234 are not within the conserved domains that have been described, Greenblatt et al identified mutants in 3 additional regions within codons 151 through 164, particularly...
in all 21 VSS, suggesting that p53 mutation was not preexisting in these cats. People with Li-Fraumeni syndrome (a familial predisposition to several different tumor types including sarcomas, breast cancer, and brain tumors) have germline mutations involving 1 allele of the p53 gene that predisposes to tumor development if the second (normal) allele undergoes somatic mutation.\textsuperscript{10,11} The findings in our study of 21 VSS do not support germline p53 gene mutation as a cause of increased tumor susceptibility in the cats with VSS.

Because detection of mutations in fixed tissues using PCR and nucleotide sequencing is a laborious and expensive process, indirect tests including IHC using PCR and nucleotide sequencing is a laborious and expensive process, indirect tests including IHC may be used to predict mutations.\textsuperscript{8} Therefore, it is useful to establish the relationship between p53 nucleotide sequence and p53 protein detection by IHC. There was very good, but not complete, agreement between nucleotide sequencing results and prediction of p53 mutation on the basis of IHC staining in the 21 tumors evaluated; 3 tumors had high p53 expression but mutations were not detected in these tumors (Table 2). This lack of perfect agreement may be because the entire protein-coding region of the p53 gene was not sequenced. Because 87% of p53 mutations in human tumors are found within exons 5 through 8,\textsuperscript{8} only these regions were analyzed in our study. Restriction of genetic evaluation to exons 5 through 8 may underestimate the prevalence of p53 mutations by greater than 20%.\textsuperscript{6} The p53 gene mutations may also be underestimated if mutations are in the noncoding regions of the gene.\textsuperscript{8} The p53 gene could be mutated such that no protein is produced. Results of immunohistochemical staining in such a tumor would be indistinguishable from unaffected tissue. Mutations were not identified within key p53 gene regions in any of the tumors with faint or no immunohistochemical staining in our study, suggesting that the p53 protein product was normal in these tumors.

Polymorphism at codon 163 in the p53 gene of clinically normal cats has been reported.\textsuperscript{12} Results of our study further support the existence of polymorphism at codon 163. Two superimposed bases representing different codons for the same amino acid were detected in the nucleotide chromatograph at p53 codon 163 in nonneoplastic and neoplastic tissue from 6 cats. This p53 heterozygosity may be related to the inheritance of different alleles (TAT and TAC at codon 163) from each parent.

Results of our study indicate very good, although not perfect, agreement between detection of p53 protein by IHC and existence of p53 gene mutations. In nonneoplastic tissue and within the gene regions examined, p53 mutations that may have predisposed to oncogenesis were not found. However, in 5 of 21 VSS examined, p53 mutation may have played a role in tumor progression. These findings do not preclude the existence of genetic factors, other than p53 mutation, which may predispose a subpopulation of cats to VSS.

\textsuperscript{8}Nambari P: Immunohistochemical and mutational analysis of the tumor suppressor gene p53 in feline vaccine-site associated sarcomas. Saskatchewan, SK: University of Saskatchewan, 1999.
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\textsuperscript{10}Animal Reference Pathology, Salt Lake City, Utah.

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