Somatic alterations of the *p53* tumor suppressor gene in vaccine-associated feline sarcoma

Nilanjana Banerji, PhD, and Sagarika Kanjilal, PhD, PhD

Objective—To determine somatic alterations in *p53* in vaccine-associated feline sarcoma (VAFS).

Animals—27 domestic shorthair cats undergoing first surgical treatment for primary VAFS with no history of chemotherapy or γ radiation.

Procedures—Sequence analysis was performed on the genomic sequence of *p53* (between exons 5 through 9) from tumor and blood samples obtained from the cats. Cats were monitored for 3 years and disease-free intervals and survival times calculated.

Results—Eight single nucleotide polymorphisms were detected within the genomic sequence of *p53,* with 20 of 27 cats (74%) having heterozygosity at ≥ 1 polymorphic site. Somatic loss of heterozygosity at *p53* was detected in the primary tumors of 12 of these 20 (60%) cats. Such allelic deletion was significantly associated with rapid tumor recurrence and reduced overall survival. Point mutations were rare, occurring in 3 of 27 primary tumors. The finding of malignant cells in the surgical margins was significantly associated with disease recurrence, but clear margins (with no detectable malignant cells) were not predictive of positive outcome.

Conclusions and Clinical Relevance—*p53* status is an indicator of postsurgical recurrence and overall survival in cats with VAFS. Careful follow-up is important in treating vaccine-site tumors containing allelic deletion of *p53,* whereas aggressive surgical treatment may be sufficient to control primary vaccination site tumors without the allelic loss. (*Am J Vet Res* 2006;67:1766–1772)

Abbreviations

VAFS	Vaccine-associated feline sarcoma
DFI	Disease-free interval
LOH	Loss of heterozygosity
SNP	Single nucleotide polymorphism
RR	Relative risk
CI	Confidence interval

Clinical observations and epidemiologic analyses conducted for more than a decade have established the existence of a strong relationship between vaccination in cats and development of soft tissue sarcomas at the injection sites.[1–6] For the cervical and interscapular regions, where vaccines were usually administered in the past, the risk of tumor development is 50% higher for cats receiving a single vaccination, compared with cats not receiving any vaccines.[4] This risk further increases to 127% with 2 vaccinations and to 175% with ≥ 3 vaccinations at the site. Although the actual incidence is not known, it is estimated that between 3 to 13 cats develop VAFS for every 10,000 vaccines administered.[7,8] Because most of the household cat population in the United States (which is currently > 73 million) receives ≥ 1 vaccination each year, a sizable number of cats are at risk of developing this disease.[9]

Vaccine-associated feline sarcomas are highly invasive tumors with poorly defined margins.[5,6,10–14] The tumors are predominantly fibrosarcomas and malignant fibrous histiocytomas, although other histologic subtypes have occasionally been reported.[6,10,11] These aggressive tumors are characterized by severe pleomorphism, high mitotic indices, and central areas of necrosis. The recurrence rate after surgery has been estimated at > 60%, with most such recurrences appearing within 6 months of surgical excision of the primary tumor.[5] Distant metastases (primarily to the lung) are also detected in 28% of affected cats within approximately 2 years of diagnosis of the primary tumor.[14,15] Not surprisingly, early detection and aggressive excision of tumors at referral hospitals lead to a better outcome, extending the median DFI from just 66 days to well over 300 days.[15,16]

The purpose of the study reported here was to determine somatic alterations in *p53* in cats with VAFS. The *p53* tumor suppressor gene plays an important role in cellular homeostasis.[17] Upregulation and stabilization of the p53 protein in cells with DNA damage lead to cell cycle arrest in the G1 phase, thereby allowing various repair enzymes to restore the genomic integrity. The protein also serves as a switch, sending cells with excessive or irreparable damage into a process of programmed cell death. Somatic alterations of *p53* are frequently found in tumors of human and animals and can lead to an aggressive phenotype.[17,18] Because somatic alterations of the *p53* tumor suppressor gene are frequently associated with tumor aggressiveness, we have analyzed the most commonly affected regions of the gene by automated DNA sequence analysis in 27 cats with VAFS. We here present strong evidence for LOH at this locus in vaccine-site tumors and further show a significant negative association of such allelic deletion with time to local disease recurrence and overall prognosis.

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From the Department of Veterinary PathoBiology, College of Veterinary Medicine (Banerji, Kanjilal), and the BioMedical Genomics Center (Kanjilal), Academic Health Center, University of Minnesota, Saint Paul, MN 55108. Drs. Banerji and Kanjilal's present address is Department of Medicine, Medical School, University of Minnesota, 1500 Gortner Avenue, Saint Paul, MN 55108.
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Address correspondence to Dr. Kanjilal.

Materials and Methods

Animals—All 27 cats with VAFS included in this study were domestic shorthair cats referred to the University of Minnesota Veterinary Teaching Hospital for surgical treatment of primary tumors. All tumors were located at sites of prior vaccination and confirmed by board-certified pathologists to have presented typical histopathologic features of vaccine-associated sarcoma.[6,10,19] Tumors were located in the interscapular or scapular area for 19 patients, whereas 8 were located on the proximal portion of a hind limb (upper portion of thigh). All of the 27 patients were undergoing their first treatment for VAFS, and thoracic radiographs available at the outset indicated that the patients were free of lung metastases at the time. Wide (3 to 5 cm) margins were maintained during surgical resection of the tumors and described as radical in 8 cats (removal of dorsal spinous processes in 6 cats and amputation in 2 cats).[15] None received adjuvant chemotherapy or radiation as part of treatment for the primary tumor. Demographics, vaccination history, and clinical details were recorded for the study.

Tissue collection and storage—Specimens of the tumors and tumor-adjacent nonmalignant tissue were taken from incidental materials generated during surgical resection of the tumors by board-certified veterinary surgeons. The tissue specimens were snap-frozen and stored at –80°C until processed for DNA extraction. Blood samples collected from the cats in evacuated tubes[a] were stored overnight (if necessary) at 4°C prior to DNA extraction.

DNA extraction—Genomic DNA was extracted from the samples by use of a commercially available kit for DNA purification.[b] Genomic DNA from the blood of a disease-free cat was purchased[c] and used as a positive control for PCR analysis.

Amplification of *p53* exons 5 to 8 by PCR assay—Segments of feline *p53* were amplified by use of 3 pairs of site-specific primers designed from the primary genomic sequence (**Appendix**; GenBank accession Nos., D26608, DQ119105, and AF175762).[20,21] Each reaction mix contained 50 ng of genomic DNA template, 0.2µM each of forward and reverse primers, 100µM deoxynucleotide triphosphates, 1.5mM $MgCl_2$, 0.75 units of *Taq*,[d] and 1X PCR buffer[e] in a total volume of 25 µL. After activation of *Taq*[d] at 94°C for 10 minutes, amplification was performed by use of the first 2 primer pairs with annealing temperatures of 65°C for 2 cycles, 60°C for 3 cycles, and 55°C for 25 cycles for the first 2 primer pairs. An annealing temperature of 55°C was maintained for 30 cycles for amplification by use of the third primer pair. Extension and denaturation temperatures were maintained at 72°C and 94°C, respectively, and all segments were 30 seconds in duration except on the last cycle, for which the extension period was increased to 5 minutes.

Nucleotide sequence analysis—Amplicons were purified by ultrafiltration[e] and sequenced[f,g] for all study cats. Sequencing reaction mixtures were electrophoresed on an automated DNA sequencer[h] at the University of Minnesota Advanced Genetic Analysis Center and sequences analyzed by use of software programs.[i,j]

Follow-up examinations—Patients were generally examined at 2 weeks and 1, 3, 6, 9, 12, 18, 24, 30, and 36 months after surgery. Owners also monitored the surgical site for lumps or other abnormalities and brought their cats to the clinic for physical examination in case of concerns in between scheduled visits. Biopsies were performed on recurrent masses for detection of malignant cells. Thoracic radiographs were performed 3 months after surgery and repeated at follow-up visits if metastatic disease was suspected. All cats were followed for at least 3 years after surgery, and cats that died within the period were subjected to necroscopic examination.

Data analysis—Disease-free intervals were calculated as the days from date of first surgery to local or distant disease recurrence (if any). Overall survival was also calculated from date of first surgery to death if the event occurred. Mean, median, and SD values were determined for demographics (such as age and weight) and tumor size. Event distribution was plotted by the Kaplan-Meier method. Data were censored for 8 cats that were in good health with no occurrence of event as well as 1 cat that died of other causes during the 3 years of follow-up. No cats were otherwise lost to follow-up. Differences in DFI for the cats that harbored a *p53* deletion (LOH-positive) and cats without the somatic loss in their tumor tissues (LOH-negative) were compared by use of a log-rank test (a nonparametric test suitable for the number of samples included in the study).

The associations between DFI and LOH, age, sex, and weight of the cats as well as tumor size, tumor site, or the time elapsed from vaccination to tumor development at the site were assessed with a Cox proportional hazards regression model with regression coefficients determined for each covariate and risk ratios calculated from the exponent of the coefficients. Analyses were performed by use of statistical software packages.[k,l] Values of $P < 0.05$ were considered significant.

Results

Animals—The study included 11 spayed female and 16 neutered male domestic shorthair cats. All 27 cats had a diagnosis of VAFS on the basis of previously described histologic features and resembled cats in larger epidemiologic investigations in their demographic information.[5,6,10] The mean age of the study population was 8.7 ± 3.1 years (range, 1.5 to 16 years). The median age was 8 years, with all but 2 cats within the range of 4 to 13 years. The mean weight of the cats was 5.6 ± 1.3 kg, (median, 5.6 kg; range, 3 to 8 kg). Cats with VAFS had a history of vaccine administration against rabies, FeLV, feline viral rhinotracheitis, calicivirus, and panleukopenia. The vaccine-site tumors developed in 55% of the cats within 1 year of vaccination, and 92% of cats had tumors by the third year. The median period between vaccine administration and tumor development at the site was 12 months (range, 1 to 72 months). On average, tumors were 4.3 ± 2.9 cm at their largest dimension (median, 3.3 cm; range, 1.5 to 19.0 cm). Margins after resection of primary tumors contained malignant cells (ie, not clear margins) for 9 cats. No malignant cells could be detected in the margins (ie, clear margins) for 10 cats; margin status was unknown for 8 cats.

Polymorphisms in feline *p53* sequence—Automated DNA sequence analysis of *p53* amplicons from samples obtained from the study cats and the control feline genomic DNA revealed the presence of 7 SNPs in addition to a previously reported polymorphism at the third base of codon 163 in exon 5 (GenBank accession Nos., DQ119105 and AF175762).[22] Of these sites, polymorphism was prevalent for the third base of codon 163 (C and T); intron 7 positions 14 (C and T) and 259 (C and T); and intron 8 positions 15 (C and T), 20 (A and G), and 70 (C and

T). Two sites, intron 7 position 12 and intron 8 position 16, mainly had alleles with C and G nucleotides (instead of T and A), respectively. In addition, insertion of a third thymidine to the existing 2 thymidines at positions 246 and 247 of intron 7 was also observed. Of the 27 cats, 7 were homozygous at all the polymorphic sites and thus were noninformative for LOH in the conserved domain of *p53*. The remaining 20 cats were classified as informative (ie, heterozygous for at least 1 polymorphic site and therefore informative for allelic loss at the locus in malignant cells).

Somatic alterations of *p53* in VAFS—Analysis of the polymorphic sites in DNA from blood samples and tumor specimens from the 20 informative group cats revealed allelic loss of *p53* in 12 tumors. In addition to LOH, point mutations in exon 5 were detected in the primary tumors from 3 cats. Of these, 1 tumor had a missense mutation at codon 179, which is located within a region of *p53* that is conserved across species.[23] The wild-type sequence at codon 179 encodes a histidine residue that plays an important role in maintaining the 3-dimensional structure of the core domain of *p53*.[23] A transversion at the first position of the codon (from C to A) with a predicted amino acid sequence change to asparagine occurred in the single retained allele in this tumor. Another tumor had a single base deletion at the third position of codon 184, leading to a series of amino acid substitutions within the evolutionarily conserved domain (from codon 184 onward) and culminating in a stop codon in exon 7. The third mutation consisted of a transition (from G to C) at the second base of codon 156 (a location that is not well conserved across species), leading to an amino acid change from cystine to serine.

Follow-up data—By 3 years (1,095 days) of follow-up, 18 of the 27 cats (66.7%) had died or been euthanatized with disease, 1 had died of other causes at 111 days after surgery, and 8 (29.6%) were alive and in good health with no disease recurrence. The 18 cats with disease relapse included 2 cats that had metastasis (145 and 677 days after surgery, respectively), 3 cats that developed local recurrence as well as metastases, and 13 that had local disease only. Thus, the overall rates of metastasis and local recurrence for cats in this study were 18.5% and 59.3%, respectively. All 5 cats with metastasis were euthanatized at the time of detection of lung involvement, whereas 8 of the 13 cats with only locally recurrent disease received further surgical treatment (along with adjuvant treatment with γ radiation in 3 cats and intralesional injection of carboplatin in 1 cat).

Impact of allelic deletion of *p53* on outcome—Allelic deletion in *p53* in the primary tumor had a negative impact on outcome as shown by the Kaplan-Meier plot (**Figure 1**). Cats that were LOH positive in the primary tumor tissue rapidly developed recurrences (median DFI, 196 days; range of DFI, 39 to 311 days). Of these 12 cats with LOH-positive primary tumors and disease recurrence, 3 had developed local and distant disease at the time of recurrence, whereas 1 developed distant metastases without signs of local recurrence. Log-rank analyses indicated significant differences in DFI between the LOH-positive group and cats that were either LOH negative ($P < 0.001$) or noninformative for LOH ($P = 0.010$). Five of 8 cats in the LOH-negative group had no relapse after surgery at > 3 years of follow-up, 2 developed local recurrences at 315 and 292 days after surgery, and 1 cat developed metastasis detected at 677 days after surgery. Although all the primary tumors in this group were LOH negative, the tumor that recurred at 292 days (the shortest DFI in this group) contained the mutation at codon 156 and later underwent allelic loss of *p53* in the recurrent tumor. In the noninformative group, 3 of 7 cats had no relapse after surgery at > 3 years of follow-up, 1 died of other causes at 111 days into the follow-up period, and 3 developed local recurrences (at 46, 105, and 492 days, respectively).

As with DFI, a significant negative association was also found between occurrence of LOH in the primary tumor and overall duration of survival (Kaplan-Meier plot not shown). At 3 years of follow-up, 14 of 20 informative group cats (12 of 12 cats from the LOH-positive group and 2 of 8 cats from the LOH-negative group) had died or had been euthanatized with disease. Cats in the LOH-positive group had an overall median survival of 223 days (range, 51 to 730 days). Survival was significantly (log-rank test, $P < 0.001$) longer for cats that did not harbor LOH in their primary tumors, with 6 of the 8 cats in this group alive at > 3 years. Two cats in this group had been euthanatized, 1 at 677 days because of development of metastasis and 1 at 748 days because of aggressive local recurrence of the tumor after a second surgery with adjuvant radiation treat-

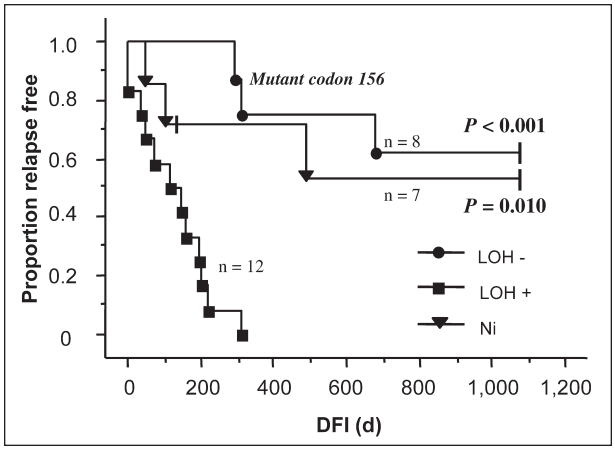

Figure 1—Kaplan-Meier distribution curves comparing outcomes on the basis of *p53* status of primary tumors. Cats with allelic deletion of *p53* in their primary tumors are represented as LOH positive, whereas cats with no allelic deletion are grouped as LOH negative. Cats for which LOH could not be evaluated are presented as a separate group marked as noninformative for LOH. The DFI was calculated as the days from date of first surgery to local or distant disease recurrence (if any). Differences in DFI between the LOH-positive group cats and LOH-negative and noninformative group cats were significant ($P < 0.001$ and $P = 0.010$, respectively). Hatch marks represent censored data for 8 cats (5 in the LOH-negative group and 3 in the noninformative group) that were in good health with no occurrence of event by the end of the 3-year follow-up period, as well as 1 cat (in the noninformative group) that died of other causes during the 3-years of follow-up. LOH – = LOH negative. LOH + = LOH positive. Ni = Noninformative.

ment. The primary tumor from this latter cat contained *p53* mutation at codon 156, and the first recurrence was diagnosed at 292 days after the initial surgery. Survival was also significantly (log-rank test, P = 0.030) longer in the noninformative group, compared with the LOH-positive group. In the noninformative group, 3 of 7 cats were alive with no disease at 3 years past initial surgery, 1 died of other causes before the end of the follow-up period, and 3 with locally recurrent disease were euthanatized (at 110, 492, and 546 days after initial surgery, respectively).

The significant differences in overall survival between the LOH-positive group and the LOH-negative or noninformative groups were mainly attributable to the presence of cats with no disease recurrence in the latter 2 groups and could be detected despite the additional treatment provided to the 8 cats with recurrent disease in the study (which may have affected survival in those cats). Although the number of cats with recurrent disease receiving additional treatment was not enough to allow evaluation of the different treatment modalities, successive treatment for recurrent disease produced a significantly (log-rank test, P = 0.034) better outcome for the 2 LOH-negative cats than the 6 that belonged to the LOH-positive and noninformative groups. One cat receiving treatment for recurrent disease in the LOH-negative group achieved 414 disease-free days before the second relapse, whereas another cat in this group was healthy and disease free at the end of the 3-year period. In contrast, successive treatment resulted in much shorter times to second recurrence in LOH-positive and noninformative groups (median, 52 days; range, 24 to 122 days).

Impact of other factors on disease outcome—— Although cats in the LOH-positive group had larger median values for age, weight, tumor size, and time elapsed from vaccination, regression analysis by the Cox proportional hazards model indicated no significant associations between these parameters or sex and disease relapse (age, RR = 1.03, 95% CI of 0.83 to 1.28, P = 0.767; weight, RR = 1.18, 95% CI of 0.8 to 1.75, P = 0.387; tumor size, RR = 1.32, 95% CI of 1.06 to 1.66, P = 0.078; time from vaccination, RR = 1.02, 95% CI of 0. 34 to 1.29, P = 0.842; sex, RR = 2.36, 95% CI of 0.49 to 11.36, P = 0.290). Adding whether the surgical procedure was described as radical or not and tumor location (described as scapular area or proximal portion of hind limb) to the analysis covariate did not provide indication of any significant association. However, the strong association between LOH in the primary tumor and disease relapse was confirmed among the group of covariates (RR = 42.35, 95% CI of 4.38 to 409.5, P = 0.001). No significant relationship was detected between LOH status and margin information. Although a significant (Cox proportional hazards regression model, P = 0.022) association with shorter DFI could be detected when malignant cells were present in the margins, DFI bore no significant association with margins when they were thought to be devoid of malignant cells. Thus, although disease relapse could be predicted correctly in 8 of 9 cats by margin information when margins contained malignant cells, 4 relapses occurred in the 10 cats that were thought to have clear margins. Because tumor size may have impacted the ability to obtain margins devoid of malignant cells, we further examined the subset of 13 cats for which the tumors were ≤ 3 cm (median, 2 cm; range, 1.5 to 3.0 cm) in their largest dimension. Information on margins could be retrieved for 9 of these cats but did not correspond with outcome in 4 instances. Three cats with apparently clear margins had tumor recurrence; 1 cat in which malignant cells were thought to be in the margin had no tumor recurrence. *p53* status could be determined in the primary tumors for 10 cats and correctly predicted disease outcome in each instance.

Discussion

To begin to elucidate the molecular pathogenesis of VAFS, we have analyzed primary tumors in cats with VAFS for somatic alterations in *p53*. The evolutionarily conserved domain of this tumor suppressor gene is the most frequent site of somatic mutations in various cancers including sarcomas of the soft tissue.[23–26] Analysis of the genomic sequence of feline *p53* revealed the presence of 8 polymorphic sites. These included a previously identified SNP at codon 163 of exon 5, 3 sites in intron 7, and 4 sites in intron 8. Such intronic polymorphisms of *p53* are also present in other species, and while the functional importance of individual bases at each of the sites is not known, the presence of distinct bases at these sites (on each of the 2 copies of the gene normally present in an individual) helps to distinguish the alleles from each other.[24] Hence, comparison of sequence information at polymorphic sites in DNA obtained from matched sets of malignant and nonmalignant tissues from individual patients enables detection of allelic deletions in the malignant cells through measurement of LOH at each locus. Because individuals may be homozygous (ie, noninformative for LOH) at some of the polymorphic sites, analysis of multiple sites increases the chances that > 1 site will be informative.

Results of our study on polymorphic sites in primary tumors of cats with VAFS provide strong evidence for the involvement of allelic deletion at *p53* in the malignant progression of VAFS. Sixty percent (12/20) of the informative group cats had undergone the allelic deletion (as evidenced by LOH) in their primary tumors. The presence of LOH was found to be associated with a significantly (log-rank test, P < 0.001) increased frequency of disease recurrence after aggressive surgical treatment as well as significantly (log-rank test, P < 0.001) shorter overall survival. In the LOH-positive group, 6 of 12 cats relapsed by 4 months, 9 of 12 cats did so by 6 months, and all 12 cats had relapsed by 10 months. In contrast, only 3 of 8 cats had recurrence in the LOH-negative group: 2 had local recurrences at about 10 months after surgery, and the third had distant metastasis at > 22 months.

Significant differences in DFI (log-rank test, P = 0.010) and survival (log-rank test, P = 0.030) were also found between the LOH-positive group cats and noninformative group cats. It is possible that the 3 cats from the noninformative group that had recurrent dis-

ease also harbored *p53* deletions in their primary tumors but that these alterations could not be detected through examination of the SNP sites located within the p53 genomic sequence between exons 5 through 9. This possibility is supported by our observation that the DFIs were similar after a second treatment for recurrent disease for cats from noninformative and LOH-positive groups. Expansion of the feline *p53* SNP repertoire in the future could help in reducing the number of cats that are noninformative for LOH.

Missense point mutations were detected in only 3 of the 27 primary tumors examined by sequence analysis of exons 5 through 9 of the *p53* tumor suppressor gene. All 3 of these cats serve as classic example of Knudson's theory of inactivation of both copies of a tumor suppressor gene in malignant cells.[27] In 2 instances, alterations occurred on the single *p53* allele retained in aggressively growing tumors that had undergone LOH. In the third instance, the point mutation appeared first (in the primary tumor) and was followed by deletion of the other allele in the corresponding recurrent tumor. The presence of somatic point mutations of *p53* has also been reported by other investigators in 5 of 8 tumors chosen from a larger set of 40 unclassified vaccine-site tumors on the basis of nuclear immunohistochemical staining for *p53*.[28]

Overall, results of our study indicate that in VAFS, the first allelic inactivation of *p53* is likely to occur through deletion and that this may be followed by inactivation of the remaining allele through accumulation of point mutations. Occasional point mutations of *p53* are also capable of leading to a more aggressive tumor phenotype, but this is likely dependent on the specific location of the point mutation and the type of change it brings about in the translated protein. Although it is possible that other mechanisms, such as amplification or enhanced translation of *Mdm2*, or gross rearrangements outside the conserved regions are also involved in the inactivation of *p53*, loss of 1 allele appears to be a crucial switch (or at least marker) associated with acquisition of invasive characteristics and malignant progression of VAFS.[26,29] It has been our observation that malignant cells with reduced or altered tumor suppressor activity can invade deep into the surrounding tissue; even a minimal number of such cells remaining after surgery may multiply in the environment of inflammation and wound healing at the site and rapidly grow into the recurrent tumor.[30–32]

Results of our study further indicate that although the presence of malignant cells in the margins after surgical resection of primary tumors is a significant (Cox proportional hazards regression model, $P = 0.023$) predictor of negative outcome, tumors resected with apparently clear margins can recur as well and that such recurrence may be predicted by a knowledge of *p53* status of the tumor. This lack of significant association between completeness of excision and outcome has also been found in at least 2 other larger studies (based on 78[33] and 92[34] cats with VAFS, respectively). The study[33] on 78 cats with VAFS revealed that recurrence rate or survival was not associated with completeness of excision regardless of whether the margin information was based on the surgeon's impression or results of histologic evaluation. Also, in the study[34] on 92 cats with VAFS, local recurrences were found to develop in 42% of cats with complete excision of tumors (ie, with clear margins). Results of our study further indicate that knowledge of *p53* status is important even for the management of relatively small tumors. For the 13 cats included in our study in which the primary tumors were ≤ 3 cm (median, 2 cm) in their largest dimension, *p53* status was a far more accurate predictor of disease outcome than margin information. It is possible that the initiation of malignant progression to an invasive phenotype through allelic deletion of *p53* may occur relatively early and before the appearance of severe morphologic changes in the tumor cells.

Finally, results of our study indicate that the analysis of *p53* status in primary tumors provides critical information for the management of VAFS irrespective of tumor site. Unlike *p53* status, the tumor site per se (scapular or proximal portion of the hind limb) did not significantly affect outcome in our study, wherein all tumors were removed with an aggressive, well-planned surgery at first chance. In a previous study[15] aimed at identifying populations of cats that might be effectively treated with surgery alone, it had appeared as if tumors on the limbs were associated with a significantly longer time to first recurrence (median, 325 days) than tumors of the trunk (median, 66 days); however, as clearly pointed out by the authors of that study, such comparisons were not possible because the tumors located on the limbs underwent radical excision (amputation), whereas the tumors that were located on the trunk were mostly removed with marginal first excisions performed prior to referral of the patients to an advanced veterinary center.

During the last 15 years, vaccination has been etiologically linked with the development of aggressive sarcomas in cats. Although the disease develops in 1 to 13 instances for every 10,000 vaccines administered, the poor prognosis of the disease has caused great concern to veterinary health providers and pet owners. Unfortunately, and despite the great interest in the field, not much information has been available on the molecular pathogenesis of the disease.[35] Aggressive surgery at first chance at a well-equipped hospital is known to be the most effective mode of treatment, whereas adjuvant treatment with γ radiation as well as various chemotherapeutic agents is also often used in efforts to better manage the disease.[15,35–38] However, the efficacy of continued treatment or the need for such treatment among different groups of cats with VAFS is not known.[15] Results of our study indicate that in the subset of cats without allelic loss of *p53* in their tumors, aggressive surgical treatment could provide a positive prognosis in absence of additional treatment. Our results further indicate that cats with *p53* alterations in the primary tumor are likely to have recurrences within a year despite aggressive surgical resection. The prognosis for such affected cats is likely worse when the *p53* alteration consists of allelic deletion, a relatively common event in the pathogenesis of VAFS. Although the efficacy and benefit of various modalities of continued treatment of recurrent disease

are not yet known for VAFS, evaluation of *p53* status in primary tumors may be a useful tool in screening for cats that are likely to have any added benefit from additional intervention.

As genes that play a role in the molecular pathogenesis of VAFS get elucidated (as is beginning to happen for various human sarcoma subtypes), additional markers and molecular targets could be developed for individualized treatment and improved management of the disease.[39,40] In the interim, early detection of the disease, prior to the onset of *p53* alterations, through vigilant monitoring of injection sites likely provides the best means to a positive outcome. It is likely beneficial to also continue the practice of not vaccinating in the scapular region because of the practical limitations of performing surgical excision of tumors with wide margins at the site. Furthermore, efforts and resources are necessary to perform large well-planned studies wherein the large number of clinical parameters that affect outcome (such as specifics of treatment subgroups, use of comprehensive diagnostic tools, and follow-up processes used) can be carefully controlled and analyzed. Above all, research on the subject and publication of results should be encouraged so as to help alleviate this important companion animal health issue.[19,35]

a. Vacutainer blood collection tube, Becton, Dickinson & Co, Franklin Lakes, NJ.
b. QIAamp tissue kit, QIAgen Inc, Valencia, Calif.
c. Novagen, EMD Biosciences, Madison, Wis.
d. AmpliTaq Gold DNA polymerase with GeneAmp 10× PCR gold buffer, PE Applied Biosystems, Foster City, Calif.
e. Microcon YM-100 centrifugal filtration unit, Millipore Corp, Bedford, Mass.
f. AmplitaqFS Dye-terminator kit, PE Applied Biosystems, Foster City, Calif.
g. BigDye terminator cycle sequencing kits, version 3.1, PE Applied Biosystems, Foster City, Calif.
h. ABI model 377 DNA sequencer, PE Applied Biosystems, Foster City, Calif.
i. EditSeq, DNASTAR Inc, Madison, Wis.
j. MegAlign, DNASTAR Inc, Madison, Wis.
k. StatView version 8.2, SAS Institute Inc, Cary, NC.
l. GraphPad Software Inc, San Diego, Calif.

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Appendix

Primers for PCR amplification of feline *p53* segments.

Segments	Primers	Amplicon size (bp)
Exon 5 + intron 5 + exon 6	5′-TACTCCCCTCCCCTCAACAA-3′ and 5′-CAGACCTCGGGCGGCTC-3′	386
Exon 7 + intron 7 + exon 8	5′-GTCGGCTCTGACTGTACC-3′ and 5′-CTTACCTCGCTTAGTGCTCC-3′	519
Intron 7 (partial) + exon 8 + intron 8 + exon 9 (partial)	5′-CTTTGGGACCTTCTCTTACC-3′ and 5′-ATTCTCCATCCAGTGGCTTC-3′	418