Association of Germ-line Polymorphisms in the Feline \( p53 \) Gene with Genetic Predisposition to Vaccine-Associated Feline Sarcoma

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Abstract

A case–control study was conducted in order to investigate the association of polymorphisms in the genomic sequence of the feline \( p53 \) gene with a predisposition to vaccine-associated feline sarcoma (VAFS). In the study, 50 domestic short hair cats with a confirmed histopathologic diagnosis of VAFS were matched to disease-free controls (1:2) by age, sex, and breed. Cats from both the diseased (case) and control groups were also negative for feline leukemia virus and feline immunodeficiency virus. Germ-line DNA was prepared from blood samples from cats in both groups and analyzed for sequence variation at 8 polymorphic sites in the \( p53 \) gene. A strong association was found between VAFS and the presence of specific nucleotides at 2 of the polymorphic sites. The strongest association was observed for a single-base insertion in intron 7 of the gene with an odds ratio of 8.99 (95% confidence interval = 3.42–23.57, \( P \), 0.0001). The results of the study indicate that analysis of the presence or absence of the identified genetic markers in apparently healthy disease-free cats may help in predicting which individual animals are at greater risk of developing the disease.

Vaccine-associated feline sarcoma (VAFS) was first reported in domestic cats in 1991 after a sudden increase in the number of fibrosarcomas diagnosed at vaccination sites as compared with other locations (Hendrick and Goldschmidt 1991). A substantial increase in incidence of the vaccination site tumors was found to have occurred between 1988 and 1994 after the enactment of compulsory vaccination laws in many states (Hendrick et al. 1994; Doddy et al. 1996). Subsequent epidemiologic studies helped establish a causal relationship between the administration of vaccines and the development of tumors at the injection sites, with risk of tumorigenesis increasing by 50% with a single vaccination, 127% with 2 vaccinations at a site, and 175% when 3 or more vaccinations are simultaneously administered (Kass et al. 1993).

The mechanism of tumorigenesis at vaccination sites has been the subject of much debate. Although the initial epidemiologic studies examined tumor development at sites of vaccination against feline leukemia virus (FeLV) or rabies, subsequent reports indicated that administration of other vaccines could also lead to tumor development (Kass et al. 1993). It has been observed that the tumors are usually surrounded by inflammation and may be preceded by adverse injection site responses (Doddy et al. 1996; Macy and Hendrick 1996). Indeed, the importance of inflammation in tumor development and progression is well established for fibrosarcoma and other malignancies (O’Byrne and Dalglish 2001). Aluminum (from the aluminum hydroxide or aluminum phosphate adjuvants present in some vaccines) was observed in macrophages within the vaccine-associated sarcomas and was initially thought to play an important role, but vaccines containing nonaluminum adjuvants and even nonadjuvanted vaccines have also been associated with the development of the disease (Hendrick et al. 1992; Ogilvie and Moore 1995). Although some preparations of rabies vaccines have been shown to be mutagenic in vitro, specific vaccine components, formulations, or brands have not been definitively implicated in the development of VAFS (Hendrick et al. 1994; McEntee and Page 2001). Possible involvement of viral oncogenes (suggested due to the rapid growth rate of the tumors) has also been proved unlikely as immunohistochemical and molecular evaluations have failed to detect feline lukemia virus, feline sarcoma virus, feline...

The high incidence of fibrosarcoma in domestic cats, their relatively low age at incidence, and a number of other factors suggest the possibility of a genetic predisposition for fibrosarcoma in this species. These factors include observations of tumor development in cats following other types of inflammatory injuries such as ocular trauma (Woog et al. 1983; Dubielzig 1984; Dubielzig et al. 1990); anecdotal reports of fibrosarcoma development at locations where other drugs or vitamins have been injected (Esplin et al. 1999); observations of tumor development at suture-sites and nonhealing wounds (Buracco et al. 2002); occasional development of tumors in multiple related members; and the occasional development of multiple tumors in individual cats. Because cancer is a complex disease with both hereditary and environmental factors contributing to etiology and disease progression, it is possible that both germ-line predisposing factors (genetics) and inflammation at the vaccination site (environment) enhance the development of VAFS. As a first step to testing this hypothesis, we have examined polymorphic sites in feline \( p_{53} \) for possible association with a predisposition to VAFS. We here present the results of our investigation that demonstrate the existence of strongly associated germ-line markers of predisposition to the disease.

Materials and Methods

Animals

The matched case–control study included 150 domestic short hair (DSH) cats consisting of 50 “case” animals diagnosed with VAFS at the University of Minnesota Veterinary Teaching Hospital per previously established criteria (Doddy et al. 1996; Couto et al. 2002; Vaccine-Associated Feline Sarcoma Task Force 2005; Banerji and Kanjilal 2006) and 100 “control” cats that were disease free at the time of the study. All cats were client-owned pets from distinct households, and the medical histories of both case and control animals were ascertained from their veterinary records. Tumors in all case animals were located at sites of prior vaccination and confirmed to be VAFS per their histopathology reports. The cases comprised 29 male and 21 female cats and tested negative for FeLV and FIV. Cats in the control group were matched 2:1 to each VAFS case by age (+/−2 year) and sex (neutered male or spayed female). All control cats were also known to be FeLV/FIV negative and had also been vaccinated routinely per their veterinary records but had not developed any tumors at the injection sites. The study utilized incidental blood samples from the cats and did not involve experimental manipulation or clinical treatment of live animals.

Genotyping

Blood samples collected from the cats were used to prepare genomic DNA using a commercially available kit (QIAamp 96 DNA Blood Kit, QIAGen Inc., Valencia, CA). Segments of the feline \( p_{53} \) gene including exon 5 and introns 7 and 8 were amplified using a previously published protocol (Banerji and Kanjilal 2006). Amplified fragments were filtered through Microcon-YM100 (Millipore Corporation, Bedford, MA) and sequenced using AmpliTaqFS Dye-terminator chemistry and BigDye terminator v3.1 (Applied Biosystems, Foster City, CA). The sequencing reaction mixtures were electrophoresed on ABI model 3100 DNA sequencers (Applied Biosystems) at the University of Minnesota Biomedical Genomics Center (http://www.bmgc.umn.edu/). Sequences were analyzed using EditSeq and MegAlign software programs (DNASTAR Inc., Madison, WI) to ascertain the genotypes at 8 polymorphic sites previously identified in the amplified segments (Figure 1, Banerji and Kanjilal 2006, GenBank accession numbers DQ119105 and AF175762). As shown in the figure, these polymorphic sites (named as figure 1. Schematic representation of 8 polymorphic sites in the genomic sequence of feline \( p_{53} \). The relative positions of SNP sites 1 through 8 are shown in relation to the genomic sequence of feline \( p_{53} \) exons 5 through intron 8. The sites included T/C polymorphisms at the third base of exon 5 codon 163 (SNP1), intron 7 nucleotide positions 14 (SNP2) and 259 (SNP4), and intron 8 positions 15 (SNP5) and 70 (SNP8); A/G polymorphisms at positions 16 (SNP6) and 20 (SNP7); and a single thymidine (T) insertion to the existing 2 thymidines at positions 246 and 247 (SNP3) in intron 7 (GenBank accession numbers DQ119105 and AF175762).
single-nucleotide polymorphism [SNP] 1 through 8) include 1 site in exon 5, 3 sites in intron 7, and 4 sites in intron 8. SNP sites 1, 2, 4, 5, and 8 were polymorphic for T/C; sites 6 and 7 were polymorphic for A/G; whereas SNP3 resulted from the addition of a single thymidine (T) nucleotide to the existing 2 thymidines at positions 246 and 247 of intron 7. The SNP3 alleles were accordingly termed T2 and T3 (based on the number of T nucleotides present at the site). For purposes of quality control, genotyping was repeated a second time for 10% of the DNA samples chosen at random and the sequence information confirmed to match the original results.

**Statistical Analysis**

Ages of male and female cats from the case group were compared using an unpaired t-test (InStat, GraphPad Software Inc., San Diego, CA). Genotypes and single-allele frequencies at each SNP site were recorded for both cases and controls and odds ratios (ORs) and confidence intervals (CIs) determined for single-allele frequencies at the polymorphic sites by conditional logistic regression analysis (STATA v.9, Stata Corporation, College Station, TX). Conditional logistic regression analysis was also used to calculate the ORs separately for male and female cases. Linkage disequilibrium (LD) correlation (R) values between pairs of polymorphic sites were calculated using the expectation maximization (EM) algorithm and visualized in graphic format (HelixTree software, Golden Helix Inc., Bozeman, MT). Single-point disease association was also confirmed using the interactive tree analysis mode of the software. Using the same program, a moving window analysis of the feline p53 SNPs included in this study was undertaken to identify haplotype distributions that significantly differed between cases and controls. For all statistical analyses, P < 0.05 was considered to be significant.

**Results**

**Animals**

The case-control study included 150 DSH cats of which 50 were VAFS patients with a mean age of 9.12 ± 3.31 years (median, 9.5 years; range, 1.5–17.5 years). There were 29 male cats in the case group with a mean age of 8.43 ± 3.03 years (median, 8 years; range, 1.5–16 years) and 21 female cats with a mean age of 10.07 ± 3.52 years (median, 10 years; range, 4–17.5 years). Although the mean age of the male cats with VAFS was less than that of the female cats with VAFS, this difference did not reach our stipulated level of significance (unpaired t-test P = 0.08). The mean age of the 58 matched male control cats was 8.44 ± 3.16 years (median, 8 years; range, 2–16 years). The mean age of the 42 matched female cats was 10.13 ± 3.43 years (median, 10 years; range, 3.5–18 years).

**Genotypes and Single-Allele Frequencies in VAFS Cases and Controls**

Genotypes and single-allele frequencies at the polymorphic sites for VAFS cases and controls are summarized in Table 1. Of these sites, intron 8 nucleotide position 16 (SNP6 from Figure 1) was excluded from further analyses as this position had a minor allele (A) frequency of <5% in both cases and controls (as opposed to major allele, G; Cox et al. 2005). Conditional logistic regression analysis of the data from the remaining 7 sites indicated that insertion of a T nucleotide at SNP3 had the strongest association with disease occurrence with an OR of 8.99 (95% CI = 3.42–23.57, P < 0.0001; Table 1). The T nucleotide insertion was present in 24 of the 50 (48%) cancer cases. Of these cases, 22 were heterozygous (T2/T3) for the allele and another 2 were homozygous (T3/T3). In contrast, only 10 of 100 (10%) cancer-free controls harbored this allele in the heterozygous (T2/T3) state. Homozygous occurrence of the T3 allele was not observed in the disease-free control group. In addition, there was a significant increase in allele frequency of C (0.32 vs. 0.30) with an OR of 3.42–23.57, P = 0.0010 at SNP1 for VAFS cases over healthy controls. No evidence for significant association with VAFS was obtained for any of the other positions by this method of analysis.

To analyze the effect of gender on disease association at SNPs 1 and 3, conditional logistic regression was also performed separately for male and female cases and matched controls (Table 2). The results indicated that higher ORs were obtained for female cats than male cats for both the T3 allele at SNP3 (female cats: OR = 10.57, 95% CI = 2.34–47.68, P = 0.002; male cats: OR = 7.92, 95% CI = 2.25–27.92, P = 0.001) as well as the C allele in SNP1 (female cats: OR = 7.77, 95% CI = 1.68–35.86, P = 0.009; male cats: OR = 4.43, 95% CI = 1.71–11.51, P = 0.002). However, no significant sex-based difference in susceptibility could be inferred.

Single-locus analysis was also performed using the HelixTree suite, which too identified significant association of SNP sites 1 and 3 with susceptibility to VAFS. As expected, the strongest association (Bonferroni corrected p = 1.43 × 10⁻⁵) was again observed for the T3 allele at SNP3. The split rule for this position indicated that insertion of a T in one or both alleles (i.e., heterozygous T2/T3 or homozygous T3/T3) predisposed cats to disease. The polymorphism at SNP1 was also found to be significantly associated with disease (Bonferroni corrected p = 9.40 × 10⁻⁴). Moreover, disease association, though to a lesser extent, was observed for a third site, SNP2 (Bonferroni corrected p = 1.36 × 10⁻⁴).

**LD Analysis**

The LD between the 7 SNP sites across the feline p53 gene was calculated by the EM algorithm in HelixTree (Figure 2). Each position showed correlation with one or more other positions at LD correlation R ≥ 0.3 (Barton et al. 2004; Table 3). The strongest LD was observed for SNPs 1 and 3 with R = 0.66 indicating cosegregation of alleles in these 2 polymorphic sites in DSH cats. SNP1 was also in LD with SNP7 (R = 0.33) and SNP8 (R = 0.32). Positions at LD with SNP3 included SNP4 (R = 0.44) and SNP8 (R = 0.30). The polymorphisms of SNPs 1, 2, and 3 analyzed together constituted 8 haplotypes of which 3 (each containing T3 at SNP3) showed significant association with cases. However, no
haplotype showed stronger association with VAFS in comparison to the marker at SNP3 identified by single-point analysis.

**Discussion**

The development of sarcomas at vaccination sites in cats has emerged as a disease of considerable importance in feline medicine. The aggressive nature of the tumors leads to a poor prognosis for afflicted animals and causes much concern to their owners and caregivers (Hendrick et al. 1994; Doddy et al. 1996). Local recurrences develop after surgical treatment of primary tumors in 60–67% of cases, with a majority of the recurrences appearing in less than 6 months from surgery (Hendrick et al. 1994; Banerji and Kanjilal 2006). Distant metastases (primarily to the lung) are also detected in 28% of cases within 2 years of diagnosis of the primary tumor (Macy 1999; Hershey et al. 2000). The incidence of the disease is estimated to be between 1 and 13 for every 10 000 vaccinations with more than 50% of the 73 million domestic cats in the United States likely receiving at least one vaccination each year (Wise and Yang 1992; Lester et al. 1996; Coyne et al. 1997). Thus, management of vaccine-site fibrosarcomas is a challenging task, and early detection and aggressive surgical excision of the tumors at first chance are essential for
obtaining a positive outcome (Davidson et al. 1997; Hershey et al. 2000).

The overall goal of our studies on VAFS has been to help in elucidating the molecular pathogenesis of the disease so as to develop prognostic markers and design rational strategies for prevention and intervention. In previous studies, we have described the strong association of somatic deletion in the conserved region of the \( p53 \) gene with postsurgical recurrence and decreased survival (Banerji and Kanjilal 2006). Here, we investigate the involvement of a genetic predisposition to VAFS. Existence of a genetic susceptibility to the disease has been suggested by the relatively young age at which cats develop VAFS as compared with other tumors (Doddy et al. 1996). A preexisting genetic predisposition was also suspected due to the short latency period between vaccination and tumor development (Banerji and Kanjilal 2006), occurrence of disease in multiple cats from the same litter (Martano et al. 2005), as well as development of other primary tumors in VAFS patients (Madewell et al. 2004).

In the current investigation, we have examined 8 polymorphic sites in feline \( p53 \) for possible association with a predisposition to VAFS. Analysis of single-allele frequencies at the sites indicated that an allele with a single thymidine nucleotide insertion in intron 7 (T3) bore a very strong association with VAFS (OR = 8.99, 95% CI = 3.42–23.57, \( P < 0.0001 \)). The results show that in DSH cats, this marker was present in 48% of VAFS cases as opposed to 10% of controls. Furthermore, the occurrence of the T3 allele at SNP3 in a homozygous state in 92% of marker-positive VAFS cases and occurrence in only the heterozygous state in controls suggest a potentially lethal effect of the T3 allele in the homozygous condition.

In addition to SNP3, a synonymous substitution at SNP1 was also associated with disease predisposition (OR = 3.45, 95% CI = 1.65–7.20, \( P = 0.0010 \)). For each of the sites SNP1 and 3, ORs were higher for female cats as compared with male cats. Future studies may be useful in indicating if the odds of cancer development due to presence of the predisposing allele are higher in females than in males.

Although the feline \( p53 \) markers described herein do not lead to any amino acid substitutions in the coding sequence of the gene, intronic (as well as coding) alterations of \( p53 \) have previously been described in the rare cancer predisposition syndrome in humans known as Li Fraumeni Syndrome (Srivastava et al. 1990; Avigad et al. 1997; Barel et al. 1998). Although some of the intronic alterations in predisposed humans lead to alternately spliced forms of \( p53 \), there are other instances in which the exact mechanism by which the intronic sequences are associated with the cancer predisposition are yet unknown (Peller et al. 1995). Investigation of the mechanism by which the feline marker alleles are associated with VAFS development and studies on predisposition to other cancer types are in progress.

### Table 3. LD correlation between SNP sites

<table>
<thead>
<tr>
<th>SNP site pair</th>
<th>LD correlation R&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP1–SNP3</td>
<td>0.66</td>
</tr>
<tr>
<td>SNP4–SNP8</td>
<td>0.58</td>
</tr>
<tr>
<td>SNP7–SNP8</td>
<td>0.52</td>
</tr>
<tr>
<td>SNP4–SNP7</td>
<td>0.49</td>
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<tr>
<td>SNP5–SNP7</td>
<td>0.47</td>
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<tr>
<td>SNP2–SNP4</td>
<td>0.47</td>
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<tr>
<td>SNP3–SNP4</td>
<td>0.44</td>
</tr>
<tr>
<td>SNP2–SNP8</td>
<td>0.43</td>
</tr>
<tr>
<td>SNP2–SNP7</td>
<td>0.42</td>
</tr>
<tr>
<td>SNP4–SNP5</td>
<td>0.35</td>
</tr>
<tr>
<td>SNP1–SNP7</td>
<td>0.33</td>
</tr>
<tr>
<td>SNP1–SNP8</td>
<td>0.32</td>
</tr>
<tr>
<td>SNP3–SNP8</td>
<td>0.30</td>
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</table>

<sup>a</sup> All values were significant at \( P < 0.05 \).

### Concluding Comments

Over the years, breeding practices have inadvertently promoted and sustained a large number of genetic diseases in cats and other companion animals (Vella et al. 1999). Whereas malignant transformation at vaccination sites has only occasionally been documented in humans and other species (Marmelzat 1968; Archampong and Clark 1970), the disease has emerged as a serious problem in feline medicine possibly due to the strong founder effect observed in domestic cats (Vinogradov 1997). It is likely that the increase in instances of postvaccinal tumors observed since the 1980s results from the underlying presence of a predisposing genotype in a portion of the domestic cat population together with an increase in instances of postvaccinal chronic inflammation. Although the marker T3 allele at SNP3 was found to be overrepresented in DSH cats diagnosed with VAFS in
Minnesota in the current study, this allele is also present in cats from other geographic regions as well as in other breeds of cats (Banerji N and Kanjilal S, unpublished data). Large-scale molecular genetic testing of this locus in cats may enable the rational development of strategies for preventive care and disease management of animals that are predisposed to VAFS.

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**References**


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