K-ras Activation in Non-Small Cell Lung Cancer in the Dog

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ABSTRACT

To investigate the role of K-ras mutations in canine non-small cell lung cancer, we first determined the nucleotide sequence of the normal canine K-ras gene and then examined 21 canine lung tumors for activating K-ras mutations. Canine K-ras was analyzed by direct sequencing of polymerase chain reaction products generated with oligonucleotide primers derived from the human K-ras sequence. Four nucleotide differences were found between the canine and human K-ras sequence from position 5 to 211. The deduced amino acid sequence of the canine gene was identical to that of the human. Activated K-ras alleles were detected in 5 of the 21 canine lung tumors examined. The activating lesions were point mutations, predominantly in codon 12. Of the 14 adenocarcinomas examined, 2 (14%) had K-ras mutations. Two of 5 (40%) adenocarcinomas and the only large cell carcinoma also contained activated alleles. The overall frequency of K-ras point mutation in non-small cell lung cancer (25%) is similar to that reported in human non-small cell lung cancer. We conclude that K-ras activation by point mutation is associated with, but not necessary for, non-small cell lung cancer development in the dog.

INTRODUCTION

Lung cancer is a leading cause of death due to cancer in the United States (1). Human pulmonary neoplasms can be subdivided into two major forms: non-small cell cancers and small cell cancers. The non-small cell cancers include adenocarcinomas, squamous cell carcinomas, large cell carcinomas, and adenosquamous carcinomas. Adenocarcinoma has become the most important form of lung cancer over the past 20 years with both a relative and an absolute increase in incidence rates (2). In contrast, lung tumors are rare in the dog, and indeed, in one compilation of more than 17,000 histologically confirmed neoplasms collected from veterinary schools in the United States and Canada, only 221 primary pulmonary neoplasms were described (3). Most canine lung neoplasms are categorized as adenocarcinomas (4–6).

One gene implicated in the pathogenesis of human lung cancer is c-Ki-ras-2. The K-ras gene was originally identified by its presence in the Kirsten murine sarcoma virus, an acute transforming retrovirus, and subsequently identified in the genome of mammalian cells (7, 8). A human oncogenic c-Ki-ras-2 allele was first characterized in the Calu-1 lung carcinoma cell line and was determined to be activated by a point mutation in the first position of the twelfth codon (9). Subsequent investigations revealed activating K-ras point mutations in approximately one-third of human lung adenocarcinomas (10, 11).

These initial studies of ras mutations in human NSCLC3 reported mutations only at codon 12 of the K-ras gene and only in the adenocarcinoma subtype of NSCLC. K-ras mutations occurred more frequently in lung adenocarcinomas from smokers than from nonsmokers (12). Recent analyses of the three ras genes (K-, N-, and H-) in large numbers of NSCLC specimens and cell lines demonstrated mutations in 18–36% of all tumors examined, with 73–91% occurring in the K-ras gene (13, 14). In patients with either early-stage or late-stage NSCLC, detection of a K-ras point mutation in the tumor was a negative prognostic factor (15, 16).

The frequency of K-ras mutations in primary, spontaneous neoplasms of the lung in dogs, however, is unknown. In an experimental setting, whereby pulmonary neoplasms were induced in dogs by exposure to plutonium, K-ras oncogenes were demonstrated by the NIH 3T3 transfection/transformation assay (17). Naturally occurring lung cancer in the dog provides a model of lung cancer in an outbred animal population (18). As a companion animal, the dog closely shares the human environment in the United States and, conceivably, is influenced by many of the same environmental carcinogens that induce tumorigenesis in the human. Beagles experimentally exposed to cigarette smoke develop epithelial lesions in their tracheobronchial tree similar to the histological changes in airway morphology seen in human smokers (19). Given the high incidence rate of lung cancer in the human, with associated K-ras mutations, and the apparent low incidence rate of lung cancer in the dog, it seemed appropriate to investigate the frequency of K-ras activation in the dog. Our goal was to gain additional perspective on the role of these somatic mutations in the etiopathogenesis of both human and canine lung neoplasms.

In a previous study, we detected the K-ras gene in canine tissues (20). In this study, we first determined the canine K-ras cDNA sequence by direct sequencing of PCR products generated with human sequence-designed oligonucleotide primers. We then evaluated the sensitivity of our sequencing assay. With this information, we proceeded to sequence the K-ras products from 21 spontaneously occurring canine lung neoplasms in order to determine the nature and frequency of point mutations. Here, we report that the canine nucleotide sequence in the regions of K-ras codons 12, 13, and 61 is similar but not identical to the human sequence. In addition, we detected activated K-ras alleles in 5 of the 20 canine NSCLC specimens examined.

MATERIALS AND METHODS

Tissues. Normal beagle dog spleen was collected fresh at the time of death and frozen immediately to −70°C until analyzed. Lung tumor specimens were identified by review of pathology records at the University of California Veterinary Medical Teaching Hospital for the years 1984–1990. Tumor specimens had been collected at either surgery or necropsy from dogs with spontaneous lung neoplasms or killed in a transplant model of lung cancer in an outbred animal population (18). As a companion animal, the dog closely shares the human environment in the United States and, conceivably, is influenced by many of the same environmental carcinogens that induce tumorigenesis in the human. Beagles experimentally exposed to cigarette smoke develop epithelial lesions in their tracheobronchial tree similar to the histological changes in airway morphology seen in human smokers (19). Given the high incidence rate of lung cancer in the human, with associated K-ras mutations, and the apparent low incidence rate of lung cancer in the dog, it seemed appropriate to investigate the frequency of K-ras activation in the dog. Our goal was to gain additional perspective on the role of these somatic mutations in the etiopathogenesis of both human and canine lung neoplasms.

Received 3/2/92; accepted 6/22/92.

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1 S. A. K. and V. I. C. O. were supported individually by funds provided by the Cigarette and Tobacco Surplus Fund of the State of California through the Tobacco-Related Disease Research Program of the University of California, Grants 1FT73 and 1FT20, respectively. P. H. G. is supported in part by the Mathews Foundation for Prostate Cancer Research, Sacramento, CA. This study was also supported, in part, by a grant from the Morris Animal Foundation, Denver, CO.

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The abbreviations used are: NSCLC, non-small cell lung cancer; cDNA, complementary DNA; PCR, polymerase chain reaction.

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stained with hematoxylin and eosin, and the tumor was classified in accordance with the WHO classification by a veterinary pathologist. A portion of the tissue specimen containing at least 30% tumor cells was marked on the stained slide, and the corresponding region of each 50-µm section was dissected for DNA extraction.

Cells. A normal, nonimmortalized human fibroblastic strain designated T-3891 was used as a negative control (i.e., no ras mutations) (21). The Calu-1 lung carcinoma cell line, containing both normal and activated alleles of c-K-ras-2, was used as a positive control (9).

Primers. The oligonucleotides used for PCR amplifications were designed from human ras sequences and have been previously described (Table 1) (22).

Polymerase Chain Reaction. Total cellular RNA was extracted from the spleen as previously described and stored at -70°C (20). RNA was converted to single-stranded cDNA using retroviral reverse transcriptase primed with random hexamers. For amplification of the cDNA by PCR, an upstream primer from exon 1 and a downstream primer from exon 2 were used with a thermal profile of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C for 40 cycles.

DNA was extracted from the formalin-fixed, paraffin-embedded 50-µm sections according to our previously described method (22). Primers EK369/RS54 and RS84/RS85 were used to separately amplify exons 1 and 2, respectively, from the DNA. In order to improve the amplification of formalin-degraded DNA, times for the temperature plateaus used in the PCR were increased to 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C for 40 cycles.

To produce single-stranded DNA suitable for sequencing, double-stranded PCR products were reamplified asymmetrically with only one internally nested primer (23). For this asymmetric reaction, 2 µl of RNA/PCR or DNA/PCR products were added to a new PCR reaction containing 10 pmol of an appropriate internal primer and amplified for 40 cycles. For the tumor samples, EK223 and EK370 were used to asymmetrically amplify exons 1 and 2, respectively.

For purification of asymmetric PCR products, 91 µl of the reaction mix were added to 2 ml of sterile water in a Centricon 100 (RNA/PCR products) or Centricon 300 (DNA/PCR products) microconcentrator (W.R. Grace and Co., Beverly, MA) and centrifuged at 37°C for 20 min. The remaining 9 µl of DNA was extracted from samples containing 5, 10, 15, 25, and 30% tumor cells and sequenced for confirmation.

RESULTS

The nucleotide sequence of normal canine K-ras from position 5 to 211 was determined from beagle spleen cDNA using human sequence designed oligonucleotide primers and direct sequencing of asymmetric PCR products (Table 2). Canine K-ras differed from the human sequence at codons 27, 30, 34, and 42, and differences were also noted between the dog and rat sequences (Table 3) (25, 26). These sequence differences did not resolve in any changes of amino acids at these positions.

The sensitivity of our sequencing method for detecting mutant alleles among normal alleles was determined by sequencing DNA prepared from serial mixtures of T-3891 and Calu-1 cells.

In these experiments, the cells were harvested, counted, and combined to create a total of 10⁶ cells/sample. Genomic DNA was extracted from samples containing 5, 10, 15, 25, and 30% Calu-1 cells, amplified, and sequenced. The antisense strand of the mutant allele (ACA) could be discriminated at all dilutions including 5% Calu-1:95% T-3891 cells (data not shown).

Twenty-two archival canine specimens were sequenced: one normal lung and 21 tumor specimens. Upon histological review, 20 of the lung tumors examined were malignant and one was a benign adenoma. Fourteen samples were classified as adenocarcinomas and 5 were adenosquamous carcinomas. The remaining malignant tumor was characterized as a large cell carcinoma.

Mutant K-ras alleles were detected in sequences from 5 of the 21 canine lung tumors examined (Fig. 1). The site of the point

![Fig. 1. Mutant K-ras alleles detected in canine NSILC.](image)

Table 1 Sequences of the oligonucleotide primers used for DNA/PCR and RNA/PCR amplifications

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK369</td>
<td>5'-CGGGAGAGAGCTGCTGAAA-3'</td>
</tr>
<tr>
<td>RS54</td>
<td>5'-CCTCCTGAAAAATGACTGAA-3'</td>
</tr>
<tr>
<td>EK223</td>
<td>5'-ATGCAAATTAAACTGCTG-3'</td>
</tr>
<tr>
<td>RS84</td>
<td>5'-GTGTGGATCATATTCGTCCA-3'</td>
</tr>
<tr>
<td>EK370</td>
<td>5'-GATCGGTCTTCCACCTGAC-3'</td>
</tr>
</tbody>
</table>

Table 2 Nucleotide sequence of canine K-ras from nucleotide 5 to 211

<table>
<thead>
<tr>
<th>Codon</th>
<th>Human</th>
<th>Dog</th>
<th>Rat</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>GA (A)</td>
<td>GA (A)</td>
<td>GA (G)</td>
<td>Glu</td>
</tr>
<tr>
<td>27</td>
<td>CA (T)</td>
<td>CA (C)</td>
<td>CA (C)</td>
<td>His</td>
</tr>
<tr>
<td>30</td>
<td>CA (G)</td>
<td>CA (T)</td>
<td>CA (T)</td>
<td>Asp</td>
</tr>
<tr>
<td>34</td>
<td>CC (A)</td>
<td>CC (T)</td>
<td>CC (T)</td>
<td>Pro</td>
</tr>
<tr>
<td>35</td>
<td>AC (A)</td>
<td>AC (A)</td>
<td>AC (G)</td>
<td>Thr</td>
</tr>
<tr>
<td>38</td>
<td>CA (T)</td>
<td>GA (T)</td>
<td>GA (C)</td>
<td>Asp</td>
</tr>
<tr>
<td>42</td>
<td>AA (G)</td>
<td>AA (A)</td>
<td>AA (A)</td>
<td>Lys</td>
</tr>
</tbody>
</table>

Table 3 Nucleotide differences between the dog, human, and rat K-ras cDNA sequences from codons 3 through 68 (25, 26)

<table>
<thead>
<tr>
<th>Codon</th>
<th>Human</th>
<th>Dog</th>
<th>Rat</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>GA</td>
<td>CA</td>
<td>A</td>
<td>Thr</td>
</tr>
<tr>
<td>19</td>
<td>GA</td>
<td>CA</td>
<td>A</td>
<td>Thr</td>
</tr>
<tr>
<td>26</td>
<td>GA</td>
<td>CA</td>
<td>A</td>
<td>Thr</td>
</tr>
<tr>
<td>33</td>
<td>GA</td>
<td>CA</td>
<td>A</td>
<td>Thr</td>
</tr>
<tr>
<td>40</td>
<td>GA</td>
<td>CA</td>
<td>A</td>
<td>Thr</td>
</tr>
</tbody>
</table>

Fig. 1. Mutant K-ras alleles detected in canine NSILC. The sense strand of codon 12 shows both the normal allele (ACC) and the mutant allele, ATC (A), ACA (b), and AAC (c) in three canine tumors. The sense strand at codon 61, with both normal (CAA) and mutant (CAT) alleles visible in a fourth canine tumor.
mutation was codon 12 in four specimens and codon 61 in one specimen (Table 4). Mutations occurred in three different tumor types: adenocarcinoma, adenosquamous carcinoma, and large cell carcinoma.

**DISCUSSION**

Canine genome analysis is in its infancy. Recent studies demonstrate canine equivalents of c-N-ras, c-Ki-ras, c-Ha-ras, v-myc, c-erbB-2, c-ros-1, c-yes-1, and N-myc (20, 27, 28). Due to the pathological and behavioral similarities between many spontaneous canine and human tumors, it is logical to extend investigations in molecular oncogenesis in the dog. To our knowledge, this study is the first search for activating ras mutations in naturally occurring tumors in an outbred species other than humans.

To investigate putative K-ras mutations in dog pulmonary neoplasms, it was necessary to first sequence normal canine K-ras in the region of the 12th, 13th, and 61st codons. This was accomplished by relying on the evolutionary conservation of ras protooncogenes. Human sequence K-ras oligonucleotide primers were used in PCR to generate canine products that could then be sequenced directly. We report here the sequence of canine K-ras from position 5 to 211 (Table 2). The normal beagle K-ras nucleotide sequence is found to correspond closely to the human sequence in this region. At the four positions where the dog differs from the human, the dog sequence is the same as that of the rat (Table 3). Each species, however, has a unique K-ras nucleotide sequence providing a fingerprint for identification. All differences are degenerate, and the deduced amino acid sequence of the gene for all three species in this region is identical. Our previous work with the canine N-ras gene also demonstrates identical canine and human amino acid sequences in the corresponding region (23).

Because Calu-1 is heterozygous at the K-ras locus with both a wild-type and an activated allele, the sensitivity of our assay allows the detection of 2.5% mutant alleles or 5% heterozygous mutant cells. This compares favorably with other techniques commonly used to screen for point mutations in tissue samples. A direct sequencing technique following a single PCR amplification is reported to have a sensitivity of 10% for heterozygous ras mutants, while allele specific oligonucleotide (ASO) probing is reported to have a 5–10% sensitivity (29).

In this study, 25% of the canine NSCLC specimens examined had activating K-ras point mutations. As in human NSCLC, mutations in codon 12 predominated. In all cases, the deduced amino acid substitution would result in a protein with transforming potential according to previous *in vitro* assays (30). This frequency of K-ras mutation is similar to that reported in human NSCLC (13–33%) (14). K-ras mutation, therefore, is associated with, but not necessary for, lung cancer development in both the human and the dog.

When the tumors were categorized by histologic type, the frequency of K-ras mutation in the canine NSCLC subtype adenoscarcinoma was found to be 14% (Table 5). This frequency was less than that reported for human lung adenocarcinoma (21–31%) (11, 13, 14). K-ras mutations in human lung adenocarcinomas have been strongly linked to cigarette smoking, and a lower mutation rate might be expected in dogs. Only 7% of lung adenocarcinomas derived from people without a history of smoking have been reported to contain K-ras point mutations (12).

Because owner smoking histories were not available for the dogs in our study, the influence of sidestream smoke on the development of K-ras mutations in canine lung adenocarcinomas could not be determined. In one epidemiological study, however, only a weak relationship was found between passive tobacco smoke exposure and lung cancer risk in dogs (31). Although specific carcinogens in tobacco smoke are hypothesized to be responsible for K-ras mutations in lung adenocarcinomas affecting smokers, other mutations may be associated with K-ras activation in the dog. Dogs share the human environment, and they may serve as a sentinel population for the identification of mutagens common to both species.

For the six canine lung tumors not classified as adenocarcinomas, three (or 50%) had K-ras point mutations (Table 5). In comparison, the frequency of K-ras activation in human NSCLC other than adenocarcinoma has varied widely, with frequencies of 0% and 3% reported for Dutch and Japanese studies, respectively, and 42% reported for one study in the United States (11, 13, 14). Mitsudomi *et al.* (14) have suggested that these regional differences may reflect variations in exposure to carcinogens or cocarcinogens (14). The finding of a relatively high frequency of K-ras activation in NSCLC other than adenocarcinoma in both dogs and people from the United States may support this hypothesis. Additional studies using larger sample sizes within each tumor subtype are required to further define the role of regional differences.

In conclusion, K-ras activation occurs in a subset of canine NSCLC. Despite the wide disparity in incidence of NSCLC between dogs and people, the frequency of K-ras point mutation is similar. Although species-specific factors may be responsible for mutations within each species, exposure to common environmental carcinogens may account for some of the similarities in K-ras activation seen. Mutagenic agents induce specific types of base substitutions at preferred locations, and analysis of the mutational spectrum of K-ras in large numbers of canine NSCLC may provide clues as to the origins of these mutations (32). More specific etiological information, however, might be obtained by analysis of mutational spectra in the tumor suppressor gene *p53* due to the large number of potential sites for *p53* inactivation by mutagenic agents. Thus, further examination of both oncogenes and tumor suppressor genes in canine lung neoplasms is warranted.

**REFERENCES**

CANINE ras MUTATIONS


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