Rapid detection of K-ras gene mutations in canine lung cancer using single-strand conformational polymorphism analysis

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A total of 126 spontaneous lung tumors from pet dogs were examined for K-ras mutations within exon 1 and exon 2 using a non-radioisotope single-strand conformational polymorphism analysis (SSCP) detection method on PCR products. Mutations were confirmed by direct DNA sequencing. Tumors were classified as adenomas (9), bronchioalveolar carcinomas (59), adenocarcinomas (30), adenosquamous carcinomas (16), squamous cell carcinomas (3) and anaplastic carcinomas (9). Nineteen mutations were detected in the malignant tumors: 18 occurred in exon 1 codon 12 and one in exon 2 codon 61. No mutations were present in the adenomas. The most common mutation was a G→A transition (11/19) in the second position of codon 12. Based on this study, K-ras mutations occur in canine non-small cell lung carcinomas. The frequency and type of mutation more closely matches tumors from human non-smokers with K-ras mutations than smokers. With the application of screening techniques such as SSCP, large numbers of dog tumors can be examined to provide a large animal model for comparative studies of carcinogenesis.

Introduction

Lung cancer is the most common cause of death in both men and women in the United States, with 177,000 new cases and 158,700 deaths in 1996 (1–5). The majority of cases are linked to a history of cigarette smoking by the patient. In 1991, an estimated 90.3% of the 92,000 deaths from lung cancer in men and 78.5% of the 51,000 lung cancer deaths in women were attributed to smoking (1). Although cigarette smoking is the principle risk factor for lung cancer, the mechanisms by which smoking causes cancer are still being elucidated. Among the most common genes to be altered in lung cancer are the family oncogenes, particularly K-ras. Mutations have been found in up to 30% of non-small cell lung cancer (6). The domestic dog may serve as a useful model for studies of pulmonary carcinogenesis. In an earlier study, we demonstrated the high frequency of non-synonymous K-ras mutations in canine lung cancer by direct sequencing experiments (7). In the present study, we analyzed a larger number of specimens to further define the role of K-ras mutations in spontaneous lung tumors in the dog. To expedite the analysis, we first adapted a non-radioisotope detection method using single-strand conformational polymorphism (SSCP*) analysis, which has been shown to be a rapid and sensitive method for the detection of genetic polymorphisms (8). With this technique, accurate differentiation between wild-type and mutant K-ras PCR products for exons 1 and 2 in could be detected in previously sequenced samples. This technique was then applied to screen an additional 105 spontaneous lung tumors from dogs presenting to the Veterinary Medical Teaching Hospital at the University of California, Davis (UCD-VMTH) between 1984 and 1994. Finally, we directly sequenced any PCR product found to have a change in mobility with SSCP analysis. Both the SSCP technique used to screen for canine K-ras mutations and the results with regard to K-ras mutations in canine lung tumors are reported here.

Materials and methods

Specimens

A total of 126 spontaneous canine pulmonary neoplasms diagnosed between 1984 and 1994 at the UCD-VMTH were retrieved. Tumors of non-epithelial origin were excluded from study. Tumors were interpreted to be of primary lung origin based on the morphology and the absence of primary tumors at other sites. Representative samples of these tumors were fixed in 10% phosphate-buffered formalin and routinely processed to paraffin blocks, which were then archived at 23°C.

The neoplasms were classified by a single veterinary pathologist (SMG) on the basis of their microscopic morphology using hematoxylin and eosin (H&E) stained sections and criteria from several classification schemes (9,10). In addition, sex, age, and breed of each case was recorded. The incidence was compared to all dogs seen at the UCD-VMTH during a similar time period (1985–1994). The chi-square test was used to test for significance and a P < 0.05 was considered significant.

Lymph nodes, spleen and lung tissues similarly processed to paraffin blocks from clinically healthy beagle dogs, were used as normal (wild-type) controls. Twenty-one of the tumors had previously been sequenced for K-ras mutations and served as positive and negative controls (7).

DNA extraction

Adjacent 5-µm H&E stained and 50-µm unstained sections were cut from paraffin-embedded tissues. The microtome blade was changed after each block. The H&E slide was then examined and representative viable tumor area was circled. The corresponding area on the 50-µm slide was then scraped into a 1.5-ml sterile tube using a sterile scalpel blade for each slide. DNA was extracted as previously described with the exception that the incubation with proteinase K was done for only 1 h in a heat block (11). Samples were quantitated by spectrophotometry and stored at −20°C.

Polymerase chain reaction (PCR)/single-strand conformational polymorphism (SSCP) analysis

Portions of K-ras exons 1 and 2 were amplified by PCR from 1 µg of DNA as previously described (7,12). Oligonucleotide primers are listed in Table I. Using the thermostable DNA polymerase (Taq), 1 µg of DNA was incubated in the PCR reaction for 40 cycles of denaturation at 95°C, annealing at 55°C and polymerization at 72°C for a programmable heat block (MJ Research, Watertown, MA). SK1/SK4 primers were used to amplify a 125-base pair product that corresponded to codons 1 to 35 of K-ras exon 1. SK5/SK6 primers were used to amplify K-ras exon 2 followed by a second PCR reaction using nested primers R84/R885. The final size of the exon 2 product was 112 bp and corresponded to codons 45 to 82. The PCR products were

*Abbreviations: SSCP, single-strand conformational polymorphism; NNK, 4-(methylisotosaminio)-1-(3-pyridyl)-1-butane; BHP, N-nitosobis (2-hydroxypyridyl) amine; H&E, hematoxylin and eosin; UCD-VMTH, Veterinary Medical Teaching Hospital at the University of California, Davis; PCR, polymerase chain reaction.
Patient histories and gross tumor locations were then correlated (9), adenocarcinomas (89), adenosquamous carcinomas (16), seen at the UCD-VMTH. Tumors were classified as: adenomas there was a
dogs were seen at the UCD-VMTH. Based on this population,
During a similar time period (1985–1994), 156 444 individual
VMTH between 1984 and 1994 and retrieved for this study.
A total of 126 spontaneous canine lung tumors of epithelial
Pathologic findings

**Results**

Pathologic findings

A total of 126 spontaneous canine lung tumors of epithelial origin were submitted for histologic examination at the UCD-
VMTH between 1984 and 1994 and retrieved for this study. During a similar time period (1985–1994), 156 444 individual
dogs were seen at the UCD-VMTH. Based on this population, there was a <0.08% prevalence of lung tumors from dogs
seen at the UCD-VMTH. Tumors were classified as: adenomas (9), adenocarcinomas (89), adenosquamous carcinomas (16),
quamous cell carcinomas (3) and anaplastic carcinomas (9).
Patient histories and gross tumor locations were then correlated with the microscopic findings to further subclassify the neo-

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### Table I. Sequences of the oligonucleotide primers used for DNA/PCR amplifications

| SK1: Ki-ras -2 exon 1 (upstream), 5’-GCA TATAAGCCTGCTGAAA-3’ | SK2: Ki-ras -2 exon 1 (downstream), 5’-GGAAACAGTTACCTCTTAF-3’ |
| SK3: Ki-ras -2 exon 1 (upstream), 5’-AGTACAATAACCTTGTG3’ | SK4: Ki-ras -2 exon 1 (downstream), 5’-TGTAGGGATCATATTACC-3’ |
| SK5: Ki-ras -2 exon 2 (upstream), 5’-CAGGATTCCTACAGGAAACA-3’ | RS84: Ki-ras -2 exon 2 (upstream), 5’-GTTAGTGTGGAAGACCTG-3’ |
| KS6: Ki-ras -2 exon 2 (upstream), 5’-AACCACCATATAATGGTGA-3’ | RS85: Ki-ras -2 exon 2 (downstream), 5’-ATACACAAAGAAGCCCTCC-3’ |

*Reference (7).*

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### Table II. Primer combinations used for sequencing the Ki-ras gene

<table>
<thead>
<tr>
<th>dSSDNA</th>
<th>Asymmetric ssDNA</th>
<th>Sequenced DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 SK1/SK2</td>
<td>SK4</td>
<td>SK1</td>
</tr>
<tr>
<td>Exon 1 SK1/SK4</td>
<td>SK3</td>
<td>SK4</td>
</tr>
<tr>
<td>Exon 2 SK5/SK6</td>
<td>RS85</td>
<td>SK5</td>
</tr>
<tr>
<td>Exon 2 SK5/SK6</td>
<td>RS84</td>
<td>SK6</td>
</tr>
</tbody>
</table>

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### Table III. Histologic subtype of spontaneous canine pulmonary tumors and cases with K-ras mutations at the Veterinary Medical Teaching Hospital from 1984–1994 (% = mutations/no. cases with tumor type)

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total cases</th>
<th>Cases with mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchioloalveolar adenomas</td>
<td>9</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Bronchioloalveolar carcinomas (poorly differentiated)</td>
<td>25</td>
<td>4 (16.0%)</td>
</tr>
<tr>
<td>Bronchioalveolar carcinomas (well differentiated)</td>
<td>34</td>
<td>2 (5.9%)</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>30</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>Adenosquamous carcinomas</td>
<td>16</td>
<td>8 (50.0%)</td>
</tr>
<tr>
<td>Squamous cell (epidermoid) carcinomas</td>
<td>3</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td>Anaplastic carcinomas</td>
<td>9</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

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### Table IV. Nucleotide substitution of spontaneous canine pulmonary tumors with K-ras mutations

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Nucleotide substitution (codon)</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosquamous carcinoma</td>
<td>GGT→GTT</td>
<td>12</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>GGT→GAT</td>
<td>12</td>
</tr>
<tr>
<td>Bronchioloalveolar carcinoma (poorly differentiated)</td>
<td>GGT→GAT</td>
<td>12</td>
</tr>
<tr>
<td>Bronchioalveolar carcinoma (well differentiated)</td>
<td>CAA→CAT</td>
<td>12</td>
</tr>
<tr>
<td>Squamous cell (epidermoid) carcinoma</td>
<td>GGT→GTT</td>
<td>12</td>
</tr>
</tbody>
</table>

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plasms (Table III). One-half (50.4%) of the carcinomas and all of the adenomas were of bronchioloalveolar origin. Of the carcinomas, 17.6% had squamous cell differentiation and were most likely bronchogenic in origin.

Eighty of the tumors occurred in females and 46 in males. The female-to-male ratio of dogs with lung cancer was 1.74 compared with a female-to-male ratio of 1.14 for all dogs seen at the VMTH during a similar time period ($P = 0.022$). The average age of tumor detection was 10.9 ± 2.49 years. Of the dogs examined, 71.43% were pure-breeds. Doberman pinschers had the most lung tumors (13.5%) followed by labrador retrievers (6.3%) and golden retrievers (4.8%): these three breeds of dogs accounted for 3.1%, 8% and 5.7%, respectively, during the time period examined. When corrected for total numbers of hospital accessions, four breeds of dogs appeared over-represented, namely, Doberman pinschers (430%, $P < 0.001$), Australian shepherds (300%, $P = 0.008$), Irish setter (790%, $P < 0.001$), and Bernese mountain dogs (918%, $P < 0.001$).

Primary tumor size was highly variable with an average of 6.13 ± 5.82 cm. The adenomas were all 1.5 cm or less in diameter. In 110 dogs with a distinct primary mass, most tumors were located in the right (61,8%) and caudal (66,3%) lung lobes, and 35.7% of the cases had multiple intrapulmonary tumor foci. The adenomas were all located within the peripheral lung parenchyma. Complete necropsies were done on 59 dogs. Excluding seven cases with adenomas, 28.8% of the dogs necropsied had no evidence of vascular/lymphatic invasion or metastasis, 34.6% had vascular/lymphatic or intrapulmonary spread, 13.5% had metastasis to local (mediastinal) lymph nodes and 23.1% had distant metastasis.
K-ras mutations

A shift in banding pattern was detected in 19 of the 126 samples by SSCP. No false positive cases by SSCP were detected with all band pattern shifts confirmed to be K-ras mutations by sequencing. Eighteen of these occurred in exon 1 and one occurred in exon 2. The 18 mutations in exon 1 occurred in codon 12 and the single mutation in exon 2 occurred in codon 61. All 20 tumor samples and normal lymph node, spleen and lung tissue from healthy beagle dogs that were used as controls and that had no shifts in band pattern by SSCP, had wild-type exon 1 and 2 sequences only.

The overall frequency of K-ras mutation in the malignant tumors was 16.2%. None of the nine adenomas contained K-ras mutations. The most common mutation was a G→A transition at the second position of codon 12, which accounted for 59% of the K-ras mutations detected (Table IV). The second most common mutation was a G→T transversion at the second position of codon 12, which was seen in 26% of the mutant specimens. Figure 1 illustrates the SSCP analysis results of four canine pulmonary tumors with different K-ras point mutations (right lanes) compared with the wild-type K-ras lymph node control (left lanes). When analyzed within histologic types of tumors, K-ras mutations occurred with the highest frequency in the adenosquamous carcinomas, and eight of 16 were positive (Table III). Adenocarcinomas (non-squamous) and bronchioloalveolar tumors had a lower rate (13–16%) of mutation, and anaplastic carcinomas did not contain any K-ras mutations. The histologic morphology did not correlate with any specific mutation.

There were no differences in sex, age, breed, tumor size, location or presence of multiple intrapulmonary tumor foci between dogs with K-ras mutations and dogs without mutations. Excluding the adenomas, in the 52 cases with complete necropsies, 60% of the tumors with K-ras mutations had evidence of metastasis to local (mediastinal) lymph nodes (1) or distant metastasis (2), as compared with 32% (15/47) of the tumors with wild-type K-ras that had evidence of metastasis.

Discussion

The use of naturally-occurring tumors of the lung from large outbred animals in lung cancer research has been limited, partly because of the relatively low numbers of tumors observed in most animal species. In terms of spontaneous tumors, however, the numbers of tumors observed in the dog compare favorably to the spontaneous lung cancer rate in Fischer F344 rats, B6C3F1 mice and Syrian hamsters (15). Primary lung cancer in the dog has several unique attributes as a model for lung cancer in people. Unlike laboratory animals, dogs are usually outbred animals, they often share the same environment as their owners, and have relatively long life-spans. The annual incidence rate for primary lung cancer in pet dogs was estimated in Northern California to be 4.2 per 10 000 dogs per year (16). Although these epidemiological data are now almost 30 years old, a more recent study of primary lung neoplasms in two beagle colonies revealed a crude incidence lung cancer rate of 8.8%, with the majority of tumors occurring in old dogs dying after 13.6 years of age (15).

As illustrated by the pathologic findings of this study, neoplasms from the lungs of dogs correspond best to human tumors categorized as non-small cell lung cancer. Small cell lung tumors are extremely rare in the dog, in both naturally-occurring and radiation induced tumors (15,17,18). Further, many canine pulmonary tumors are carcinomas arising in the periphery of the lung, particularly bronchioloalveolar tumors.

Although squamous cell carcinoma remains the most common type of lung cancer in people, the proportion of patients diagnosed with adenocarcinoma and adenosquamous tumors has increased significantly (2,19). From 1950 to the present, for instance, the ratio of squamous cell carcinomas to adenocarcinoma in lung cancer in males has increased from roughly 1:18 to 1:1.2–1:4 (20). Another notable change is an increase in the number of tumors that arise in peripheral airways (2). These changes have been attributed to changes in cigarette manufacturing, including the switch to filtered tip cigarettes, low nicotine formulations and an increased nitrate content, which results in deeper inhalation of higher numbers of small particle carcinogens (4).

To investigate the role of K-ras mutation in canine lung tumors, it was necessary to first adapt a simple screening method for detection of mutations. With SSCP analysis, a mutated gene sequence has a single-strand conformation differ-
ent than its wild-type sequence, which causes a change in mobility in the mutated conformation in polyacrylamide gel electrophoresis under non-denaturing conditions (21). The specific conditions necessary for separation of mutant bands by SSCP for canine K-ras were determined using five previously published mutant samples as positive controls. Optimal detection of K-ras exon 2 required a nested PCR product, a larger gel apparatus, higher voltage, longer electrophoresis and a higher TBE buffer concentration as compared with exon 1. Further optimization of our technique was obtained by providing constant temperature with a circulating water-bath. Contrary to other reports, in this study, the addition of 5% glycerol to the gel solution resulted in fainter bands than without it (22). Different mutants tended produce the same unique banding pattern consistently. In all cases, mutant samples contained bands at positions identical to the wild-type samples in addition to the mutant bands. Our findings of only wild-type sequences in the 20 specimens with normal SSCP banding patterns, and K-ras mutations in all cases with abnormal SSCP patterns, which was confirmed by sequencing, suggests that the incidence of false negative and false positive results with our technique is low.

We report here an overall frequency of 16.2% for K-ras mutations in canine non-small cell lung cancer. All of the mutations occurred in either codon 12 or 61 and corresponded to known activating mutations at those sites. It is possible that mutations may be present outside the regions of the K-ras gene examined in this study. However, this is unlikely because by far the most common mutations in ras genes have been reported at codons 12, 13 and 61 of the 188 codons making up the gene (23).

Although the overall frequency of K-ras mutations is somewhat decreased in this large study when compared with the 25% mutation rate in an earlier study of 25 canine non-small cell lung cancer specimens, many of the results are similar. The overall frequency in adenocarcinomas, for instance, was 14% in the initial 14 tumors reported and 11% in the 89 carcinosomas examined in this study (7). Similarly, two of five adenosquamous carcinomas had K-ras mutations in the original report and eight of 16 in this study. In addition, the present study includes data on nine adenomas.

Because the adenomas were all negative, K-ras mutations may occur in canine pulmonary carcinogenesis at the time of malignant transformation, which is similar to what has been shown in people (24). In addition, well-differentiated bronchioloalveolar carcinomas had a lower frequency of mutation than did poorly differentiated tumors.

In the dog tumors, the most frequently observed mutation was a G→A codon 12 transition (11/19), but a G→T transversion at the same position occurred in five of 19 tumors, and the overall frequency of G→T transversions was 31%. Clues to the cause of K-ras mutation may come from analysis of the specific type of mutation. G→T transversion, for example, is the most common change in smokers, but it is present in <1/3 of K-ras mutations from non-smokers (6,25). In a recent study of human lung cancer from non-smokers, a G→A transition in K-ras was found in 100% (11/11) of the tumors with K-ras mutations (25). Based on these findings, it is possible that canine lung tumors containing a G→A transition in K-ras are caused by carcinogens other than those that are most potent in environmental tobacco smoke. On the other hand, in rodent models, the tobacco carcinogens 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane (NNK) and N-nitroso (2-hydroxypropyl) amine (BHP) induce lung tumors when given orally or subcutaneously, and the majority of tumors contain G→A transition at position 2 of codon 12 of K-ras. These experiments demonstrate the potential for carcinogens found in cigarette smoke to reach the lung epithelium by routes other than air to result in a different mutation from what is usually seen in smokers (26–29). In people, it has been suggested that lung tumors with the K-ras G→A transition may have a better prognosis than with other mutations (30).

Although the overall frequency of 16% in the canine tumors is less than the overall 21% of K-ras mutations reported in human non-small cell lung cancer (4,6,24), these numbers may need to be evaluated within specific histologic subtypes. Some researchers believe that the majority of K-ras mutations in human lung cancer, for instance, occur in adenocarcinomas and that the actual frequency is higher than that reported (31). In the dog, the highest frequency of K-ras mutations occurred in tumors with squamous differentiation (adenosquamous or squamous cell carcinomas) with 50% of such tumors positive. This finding is in contrast to what has been shown in people where lung adenosquamous and squamous cell carcinomas have a lower prevalence of K-ras mutations than adenocarcinomas (4,30,31). This suggests that either the mechanism(s) underlying neoplasms with morphologic features of squamous differentiation are different from those that influence non-squamous adenocarcinomas or bronchioloalveolar carcinomas in the dog, or that different carcinogens are involved.

Another important variable when evaluating percentages of K-ras mutation is the smoking history of the participants. In a study that compared K-ras mutations in adenocarcinomas of smokers versus non-smokers, the frequency of mutations for smokers and former smokers was 30–32% whereas in non-smokers it was only 7% (6). As expected, dogs, with a mutation frequency of 11% in adenocarcinomas, appear similar to non-smokers.

At present, there are no clearly defined mechanisms or carcinogens in the environment that influence canine lung carcinogenesis, although primary lung tumors can be induced in the dog by radiation exposures (32). In contrast to a previous retrospective clinicopathologic study of canine lung cancer (using data collected from several veterinary schools in North America) in which a sex or breed predilection was not described (18), our hospital population of 156,444 dogs examined over a 10-year period had female dogs and four breeds (Doberman pinschers, Australian shepherds, Irish setters andBernese mountain dogs) that were over-represented within the lung cancer group. The cause(s) for spontaneous lung tumors in the dog is probably multiple, but there has been at least some claim that the risk for lung cancer is increased in dogs living in households contaminated with environmental tobacco smoke (33). The odds ratio for dogs with a smoker in the home was 1.6, which is similar to the estimated odds ratio for lung cancer among passive smokers of 1.35 (34). The overall low incidence of lung cancer in dogs may be partly attributable to effective filtration of inspired air within the nasal cavity and turbinates (33).

In conclusion, K-ras mutations occur in canine non-small cell lung carcinomas, although the frequency and type of mutation more closely matches the limited data in human non-smokers with K-ras mutations than smokers. The role of second-hand smoke in canine lung cancer still needs to be explored through careful documentations of cigarette exposure.
in dogs with lung cancer and examination of other genes such as p53. At this time, it appears that the majority of canine lung tumors have as yet undisclosed genetic abnormalities. We and others have recently described the wild-type canine sequence for the tumor suppressor gene p53, including exons 4–9 where the majority of missense mutations occur in human neoplasms (35). With the application of screening techniques, such as SSCP, large numbers of tumors can be examined for multiple genetic lesions allowing exploitation of the unique advantages that the dog provides as a large animal model for the comparative studies of multistep carcinogenesis.

References