M.S.
2005
M-T LIN

This is to certify that the
thesis entitled

COMPARATIVE ETIOLOGY OF HUMAN AND CANINE NASAL CARCINOMAS

presented by

MING-YU LIN

has been accepted towards fulfillment
of the requirements for the

MASTER OF SCIENCE degree in COMPARATIVE MEDICINE AND INTEGRATIVE BIOLOGY

Major Professor's Signature

July 6, 2005

Date

COMPARATIVE ETIOLOGY OF HUMAN AND CANINE NASAL CARCINOMAS

By

Ming-Yu Lin

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Comparative Medicine and Integrative Biology

2005

ABSTRACT

COMPARATIVE ETIOLOGY OF HUMAN AND CANINE NASAL CARCINOMAS

By

Ming-Yu Lin

Canine nasal carcinomas (CNCs) are aggressive neoplasms with poor prognosis. Treatment approaches include surgical excision and radiation therapy. But, mortality remains high and the etiology of this malignancy is poorly understood. On the other hand, many genetic and environmental factors including inactivation of tumorsuppresser gene *p16*, a susceptibility locus on human chromosome 4p15.1-q12, and Epstain-Barr virus (a human herpesvirus) have been found to play important roles in the etiology of human nasopharyngeal carcinoma (NPC). Based on the knowledge of NPC, this study was undertaken to determine the roles of these factors of NPC in CNCs. Our results ruled out the involvement of any member of the herpesvirus family and the canine chromosomal region orthologous to human Ch 4p15.1-q12 in the etiology of CNCs. However, frequent loss of *p16* expression was observed in the presence of low frequency of loss of heterozygosity (LOH). These result point to the involvement of *p16* inactivation, most likely through promoter hypermethylation, as a major contributor to tumorigenesis in CNCs.

ACKNOWLEDGMENTS

I want to thank many people for their kind advice and help then I can accomplish my research and this thesis. My wonderful mentor Dr. Vilma Yuzbasiyan-Gurkan was always very patient and kind in guiding me on my research and studies. I also want to thank her for easing my transition to life at MSU. My committee members Dr. Matti Kiupel, Dr. Chia-Cheng Chang and Dr. Barbara Kitchell gave me excellent advice and helped me a lot in my thesis. The members of the canine genetics lab, Donna Housley, Lee Alexander, and Joshua Webster, were always very nice to me and held me when I encountered some difficulties in the experiments. I also want to thank Dr. John Kruger, Dr. Roger Maes and Dr. Annabel Wise for their generous offering positive controls for the herpesvirus primers. I would also like to acknowledge the Companion Animal Fund of the College of Veterinary Medicine for funding most of my research.

Finally I want to thank my family, my mother, my father, my sister, my boyfriend, and my friends in Taiwan and in the USA. With their support, I overcame many obstacles and reached my goals. Now I can share my happiness with them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

(3) Nasal carcinoma with no p53 staining in nuclei

CHAPTER 1

INTRODUCTION

Nasal tumors account for 1-2% of all neoplasms in dogs.[4] The vast majority of these tumors (80-90%) are malignant,[4,28] and have an overall poor prognosis[1] due to their locally aggressive, commonly infiltrative behavior, but they rarely metastasize. Nasal tumors of epithelial origin are more prevalent than those of mesenchymal origin. Of these, carcinomas are the most common and comprise 60–70% of all nasal tumors,[7,30] including adenocarcinomas, transitional carcinomas and squamous cell carcinomas. Nasal carcinomas occur most frequently in middle age or older dogs; the median age of onset is 8-10 years but cases have been reported from 1-15 years old.[28] There is no sex predilection,[28,30] although one paper reported an excess risk in males.[15] Currently, breed predilection is still not very clear, but several papers suggested that dolichocephalic breeds are more likely to develop nasal carcinomas.[6,15] This is possibly due to an increased chance of exposure and trapping of carcinogens in long-length noses. Only few papers have discussed the etiology of canine nasal carcinomas. Some have suggested that environmental pollutants might contribute to tumorigenesis.[10] Indoor kerosene or coal combustion were demonstrated as risk factors in one case-controlled study.[6] No genetic factors were investigated in past studies.

Nasopharyngeal carcinoma (NPC) is a human malignancy with a high incidence in southern China and South East Asia. They occur more often in men than in women; the ratio is about 2 or 3 to 1. The age distribution is younger than other human cancers, usually occurring at 50-60 years of age.[34] Several factors, including genetic, environmental and viral infections, have been demonstrated to contribute to the etiology

of NPC. Genetic factors that are associated with an increased risk of NPC in the Chinese population include some MHC profiles, HLA alleles A2, B14 and B46.[13] Deletion on chromosome 3p and 9p are the most frequent genetic changes observed in NPC,[24] suggesting tumor suppressor genes in these regions. The most probable candidate genes for these regions are *RASSF1* and *p16*.[9,26] *p16*, also known as cyclin-dependent kinase inhibitor 2A, is a tumor suppressor gene located at human chromosome 9p21. In cell cycle, cyclin dependent kinase 4-6 (cdk4-6)/cyclin D complex phosphorylates Rb, which leads to the release of transcriptional factor E2F and promotes the cell from G1 to S phase. p16 protein inhibits the formation of cdk4-6/cyclin D complex and results in G1 arrest (Figure 1).[23,33] Loss of *p16* was first found in several human tumor cell lines derived from non-small cell lung cancer, melanoma, and leukemia.[17] Later *p16* was shown to be inactivated through deletion, point mutation or/and promoter methylation in a high percentage of human cancers, including pancreatic adenocarcinomas, head and nasal squamous cell carcinomas, melanomas, leukemia, and gliomas.[21,32] Deletion, point mutation or promoter hypermethylation of *p16* are also found in 60-80% of primary tumors in NPC cases.[24] Another important tumor suppressor gene *p53*, which plays a role in DNA repair, cell cycle arrest, and induction of apoptosis of DNA damaged cells, does not mutate as frequently in NPCs as in other human tumors and only some point mutations were reported in NPCs.[8,36] However, an increased amount of the p53 protein was observed by immunohistochemistry in one study of NPCs.[2] As wild-type p53 turns over rapidly, normal tissues do not show significant amounts of p53 staining by IHC and the presence of p53 staining is generally taken as evidence of a mutation in p53, which stabilizes the protein resulting in increased cell staining.[29] In addition, linkage to a region

of chromosome 4 (4p15.1-q12) has been demonstrated in certain families from Guangdong province, China, with LOD scores 3.54 and 4.2 for human chromosome 4 markers D4S405 and D4S3002,[11] but a specific susceptibility gene has not yet been identified.

Another important characteristic of NPCs is a strong association with Epstein-Barr virus (EBV) infection.[40] This virus belongs to the gammaherpesviridae subfamily and is a human herpesvirus found in association with several human malignancies including Burkitt's lymphoma and gastric adenocarcinoma.[37] Several genes encoded in its genome including Epstein-Barr nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP1), and EBV-encoded RNA 1 and 2 (EBERs 1 and 2) are consistently transcribed in malignant cells and their products can be detected in NPC cells.[34] The oncogenic activity of these products has been demonstrated.[19,38,42]

Several environmental factors are suspected to play a role in the tumorogenesis of NPCs, including the intake of preserved food at an early age, use of salt cured food, which includes the traditional salty diet in South China, occupational exposure to formaldehyde and wood dust, and tobacco smoke and alcohol abuse.[43]

Based on the knowledge of human NPCs, this study was undertaken to determine if any of the genetic or infectious factors identified in human NPCs are involved in the etiology of canine nasal carcinomas. We selected *p16*, human chromosome 4p15.1-q12 region, herpesviruses, in particular EBV and p53 protein expression as the targets of our investigation. We hypothesized that:

1. *p16* inactivation is a frequent event in canine nasal carcinomas.

1.a. Nasal carcinomas show loss of heterozygosity (LOH) around canine *p16* gene.

1.b. Nasal carcinomas show hypermethylation in canine *p16* promoter region.

1.c. Nasal carcinomas show reduced p16 expression as assayed by immunohistochemistry (IHC) with an anti-p16 antibody.

2. Losses on canine chromosomal regions orthologous to human Ch 4p15.1-q12 can be found in canine nasal carcinomas.

3. A herpesvirus can be found in tumor cells in canine nasal carcinomas.

4. Canine nasal carcinomas express p53 protein as assayed by IHC with an anti-p53 antibody.

CHAPTER 2

MATERIALS AND METHODS

Identification of cases of canine nasal carcinomas

The surgical pathology reports of all canine nasal carcinomas that had been submitted to the Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University over a 5 year period (1999 to 2003) were retrieved. The formalin fixed, paraffin embedded tissue blocks of these cases were retrieved from DCPAH archives. These blocks were recut and stained with hematoxilin and eosin. Tumor pathology was reevaluated. In total, 43 cases of nasal carcinomas were identified that included the following 3 tumor entities: adenocarcinomas, squamous cell carcinomas and transitional carcinomas. Of these, 30 cases including 14 adenocarcinomas, 10 squamous cell carcinomas and 6 transitional carcinomas had sufficient amounts of normal and tumor tissue and were selected for LOH studies on canine *p16* and the canine chromosomal region that is orthologous to human Ch 4p15.1-q12. All 43 tumor samples were studied for the presence of herpesvirus. The signalments of these 43 cases are listed in Table 1. The average age of affected dogs was 10 years and ranged from 4 to 16 years. Eighteen cases were male and 10 cases were female. Most of the cases were mixed breed dogs, followed by Labrador retriever.

Isolation of DNA from formalin fixed paraffin embedded tissues

The slides of each case were reviewed and the normal and tumor tissue where definite diagnosis could be made were marked for the following DNA extraction. DNA was extracted based on a method described by Banerjee *et al.*, 1995.[3] A small section of normal and tumor tissue, each around 2 x 2 x 2 mm, was excised from each block using a

scalpel blade and placed into separate 1.5 mL microcentrifuge tube. To each tube, 400 μL of digestion buffer (50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween) was added. The sample was heated at 95°C for 10 minutes and was microwaved for 30 seconds twice at full power. The sample was vortexed thoroughly at each stop point during this period. The sample was then allowed to cool down to room temperature and 5 μL of 15 mg/mL proteinase K was added and the sample was incubated at 42°C overnight. The next day, the sample was heated at 95 °C for 10 minutes to inactivate proteinase K and was centrifuged at 12,000 rpm for 10 minutes at room temperature, and a 150 μL of aliquot was transferred to a fresh microcentrifuge tube. Negative controls without tissue samples were placed every three samples. Original concentration, 10, 25, and 50-fold dilution of this preparation were used as templates in PCR reactions.

Investigation of viral presence

Investigation of the presence of 21 species of herpesviruses by universal herpes primers and PCR

A set of degenerate PCR primers that amplify 21 species of herpesviruses (8 human and 13 animal viruses)[39] (Table 2) was used to detect the presence of herpesvirus in canine nasal carcinoma samples. Twenty five microliters of PCR mixture contained 5.0 μL (1-750 ng) of template DNA, 1 μM of each primer (5'-TGTAACTCGGTGTAYGGNTTYACNGGNGT-3' and 5'-CACAGAGTCC GTRTCNCCRTADAT-3'), 80 μM (each) of deoxynucleoside triphosphate, 0.5 U of *Taq* polymerase (Invitrogen, Carlsbad, CA), 2.5 μL of 10x PCR buffer (200 mM Tris HCl (pH 8.4) and 500 mM KCl, Invitrogen, Carlsbad, CA) and 2 mM $MgCl_2$. PCR reaction was performed under mineral oil and cycled 45 times with 30 seconds of denaturation at

94°C, 1 minute of annealing at 46°C and 1 minute of extension at 72°C. Negative controls were performed every 5 samples. Viral isolates were used as positive controls. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and detected under UV light.

Investigation of presence of bovine herpesvirus 4 (BHV-4) by PCR

Bovine herpesvirus 4 is another member of the gammaherpesvirinae subfamily. It is found ubiquitously in healthy cattle and cattle with several clinical signs.[14] BHV-4 was also isolated from cats and shown to replicate in a wide variety of animal hosts including dogs.[20] The 21 species of herpesviruses that the universal herpes primers detect do not include BHV-4. For detecting the presence of bovine herpesvirus 4 (BHV-4), a heminested PCR[20] was performed as described. The first stage PCR was performed in a 25 μL PCR mixture that contained 1-2.5 μg of template DNA, 0.2 μM of primer 1 (5'-CATGACACACTATTTTAAGTACTA-3') and primer 2 (5'-ATCTTTCTTTCAGTCTCACTGTA-3'), 80 μM (each) of deoxynucleoside triphosphate, 0.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 2.5 μL of 10x PCR buffer (200 mM Tris HCl (pH 8.4) and 500 mM KCl, Invitrogen, Carlsbad, CA) and 2 mM $MgCl_2$ under mineral oil. The reaction was cycled for 35 times with 1 minute of denaturation at 94°C, 2 minutes of annealing at 59°C and 3 minutes of extension at 72°C. One microliter of the product from the first stage PCR was taken as the DNA template in the second stage PCR. The second stage PCR was performed under the same condition as the first stage PCR, but primer 2 and 3 (5'-ATAAATTTGTGAAGACAATGGGTA-3') were used. The viral DNA positive controls as well as negative water blank were used.

PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and detected under UV light.

Identification of two SNPs around canine chromosomal region that is orthologous to human Ch 4p15.1-q12

All of the linkage markers around human Ch 4p15.1-q12 were obtained from the MyScience webpage of Applied Biosystems website (https://myscience.appliedbiosystems.com/navigation/mysciMain.jsp). Each genomic sequence in the region between 46 Mb and 55 Mb on chromosome 4 was blasted against NCBI databases for canine genome (http://www.ncbi.nlm.nih.gov/genome/seq/CfaBlast.html) and two canine orthologous markers with single nucleotide polymorphisms (SNPs) were identified; a C/T variant at position 266 (in the corresponding GenBank entry) in intron 3 (GenBank accession no. X99913) of the canine rod photorecepter cGNP-gated cation channel alpha-subunit (*cCNCG1*) (GenBank accession no. X99914), which results in a change of XmnI digestion site,[41] and a G/A variant at position 265 (in the corresponding GenBank entry) of beta sarcoglycan (*SCGB*) gene (GenBank accession no. AF427093), which results in a change of HinfI digestion site (Figure 2).[5]

Identification of microsatellite markers around canine *p16*

The human *p16* sequence (GenBank accession no. AB060808) obtained from NCBI website was blasted against the NCBI databases for canine genome (http://www.ncbi.nlm.nih.gov/genome/seq/CfaBlast.html) to identify canine *p16* ortholog and its genomic neighborhood. The orthologous region was found on canine chromosome 11. All the repeat sequences around canine *p16* on canine chromosome 11: between

nucleotide 42581011 and 42617539 were acquired from the website of Ensembl Genome Browser (http://www.ensembl.org/Canis_familiaris/exportview) and three microsatellite polymorphisms: (TTTC)n, (CCA)n, and (CAAAA)n (canine Ch11: nucleotide 42599640-42599717, 42606047-42606122, and 42608679-42608710, respectively) were selected for LOH study.

Loss of heterozygosity (LOH) studies

DNA of normal and tumor tissues were isolated from the block of each case. Genotyping at the above loci were carried out using the optimum method for detection for each marker identified. For human Ch 4p15.1-q12 orthologous region, a PCR amplification fragment length polymorphism test was carried out for each SNP. For *cCNCG1*, primers were designed for a 300 bp region that contains the SNP in intron 3. PCR was performed under the same condition as described in BHV-4 section. The sequences of primers and cycling condition were summarized in Table 3. Five microliters of the PCR product was analyzed on a 2% agarose gel stained with ethidium bromide and detected under UV light. Three point five microliters of 50 mM $MgCl_2$ and 4 U of XmnI (New England Biolabs, Ipswich, MA) were added to 20 μL of the PCR product for digestion, which was carried out at 37°C overnight. The digested sample was analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and the bands detected under UV light. For *SCGB* gene, primers (5'-AACTGCATACCAATGTGACT and 5'-ATGTTCTTGATGAATTTTGGC-3') were used to amplify a 148 bp region containing the SNP site according to Brouillette JA *et al.*, 2002.[5] PCR was performed in the same condition as for *cCNCG1* followed by digestion with 4 U of HinfI (New England Biolabs, Ipswich, MA).

PCR reactions for the three microsatellite markers identified around canine *p16* were performed under the same condition as described above. The sequences of three sets of primers and the cycling conditions were summarized in Table 3. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and detected by UV light. For those samples that needed higher resolution, 6% polyacrylamide gel was used to increase separation.

Six percent polyacrylamide gel contained 6% acrylamide/bis (19:1) in 1x TBE with 10% ammonium persulfate and TEMED as catalysts. The electrophoresis was performed in a vertical gel electrophoresis device (Bethesda research laboratories) powered by 200 volts for 1 hour. The gel was stained with ethidium bromide and detected under UV light.

Identification of canine *p16* exon 1 by 5'RACE

Human and mouse INK4A/ARF loci give rise to two different transcripts, *p16* (CDKN2A) and *p14* (*p19* in the mouse). These transcripts have the common exon 2 and exon 3 but different in the first exon with *p16* having exon 1α and *p14* having exon 1β (Fig. 3).[32,35] Only a portion of the sequence of *p16* exon 2 was identified in dog (GenBank accession No. AF234176).[18] In order to identify the sequence of canine *p16* exon 1 for promoter methylation study, the sequence of human *p16* exon 1 was blasted against NCBI databases for canine genome (http://www.ncbi.nlm.nih.gov/genome/seq/CfaBlast.html) but no significant hit was found. Therefore 5' rapid amplification of cDNA ends (5'RACE) was performed to identify canine *p16* exon 1 region.

RNA extraction

RNA was extracted from dog brain tissue using the TRIzol method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Four hundred milligram of brain tissue was homogenized in 4 mL of TRIzol reagent using a glass tissue grinder. Because brain tissue has a high fat content, the sample was centrifuged at 12,000xg at 4°C for 10 minutes to separate RNA-containing supernatant from bottom extracellular material and high molecular weight DNA. The clear homogenate phase was transferred to a fresh tube and incubated at room temperature for 5 minutes. Eight hundred microliters of chloroform was added followed by vigorous mixing by hand for 15 seconds. The sample was then incubated at room temperature for 2-3 minutes and centrifuged at 4°C, 12,000xg for 15 minutes. After the centrifugation the mixtures separated into three phases. The RNA containing upper aqueous phase was transferred to a fresh tube, mixed with 2 mL of isopropanol and incubated at room temperature for 10 minutes. The sample was centrifuged at 4°C, 12,000xg for 10 minutes. After centrifugation, the RNA pellet was observed. The supernatant was removed carefully and the RNA pellet was washed with 4 mL of 75% ethanol, air-dried and redissolved in 120 μL DEPC water. The final RNA sample was analyzed to check for integrity of the ribosomal bands on a 2% agarose gel stained with ethidium bromide and detected by UV light.

DNase treatment

RNA extracted from brain tissue was treated with DNase to remove DNA contamination. The TURBO DNA-free kit (Ambion, Inc. Austin, TX) was used according to the manufacturer's instructions. Five microliters of 10x DNase buffer, 2 U of DNase and 39 μL of DEPC water were added to 10 μg of RNA to make 50 μL of final volume. The mixture was incubated at 37°C for 30 minutes. Five microliters of DNase

inactivation reagent was then added and the mixture was incubated at room temperature for two minutes. During the incubation, the mixture was vortexed for 2-3 times. The mixture was then centrifuged at 10,000xg for 1.5 minutes. The DNase inactivation reagent precipitated at the bottom of the tube. The supernatant, which contained RNA, was transferred to a fresh tube. RNA was analyzed on a 2% agarose gel stained with ethidium bromide and detected by UV light.

5' Rapid amplification of cDNA ends (5'RACE)

The 5'RACE system by Invitrogen was used according to manufacturer's instructions. Two gene specific primers GSP1 (5'-ACCAGCGTGTCCAGGAA-3') and GSP2 (5'-TGGCGGGGTCGGCACAGTT-3') were designed for this system. Both of these two primers were designed from the published sequence of canine *p16* exon 2. GSP2 is located upstream of GSP1 to increase the specificity in the final step PCR reaction.

First strand cDNA synthesis from RNA

Five microliters of DNase-treated RNA and 2.5 pmol of GSP1 in a total 15.5 μL volume was heated at 70°C for 10 minutes and was then placed on ice for 1 minute. Then 2.5 μL of 10x PCR buffer, 2.5 mM of $MgCl_2$, 0.4 mM of deoxynucleoside triphosphate and 10 mM of DTT were added. The mixture was incubated at 42°C for 1 minute and 1 μL of SuperScript™ II reverse transcriptase was added. The reaction was carried out at 42°C for 50 minutes followed by heating at 70°C for 15 minutes to stop the reaction. The sample was centrifuged for 10 to 20 seconds and was placed at 37°C. One microliter of RNase was added and the sample was incubated at 37°C for 30 minutes to digest RNA.

Purification of cDNA using S.N.A.P. column

One hundred and twenty microliters of binding solution (NaI) was added to the sample. The binding solution/cDNA mixture was transferred to a S.N.A.P. column, centrifuged at 13,000xg for 20 seconds, and the flowthrough was removed. Four hundred microliters of cold (4°C) 1x wash buffer was added to the column, centrifuged at 13,000xg for 20 seconds, and the flowthrough was removed. This wash step was repeated three more times. Then the column was washed with 400 μL of cold 70% ethanol for two times. After the wash, the column was centrifuged one more time at 13,000xg for 1 minute. The cartridge insert was transferred into a fresh 1.5 mL microcentrifuge tube. Fifty microliters of sterilized, distilled water, heated to 65°C was added to the cartridge and centrifuged at 13,000xg for 20 seconds to elute the cDNA.

TdT tailing of cDNA

A poly C tail was added to the 5' end of the purified cDNA. Ten microliters of purified cDNA, 5 μL of 5x tailing buffer and 20 μM of dCTP were added to a 0.5 μL microcentrifuge tube to make 24 μL of final volume. The mixture was heated at 94°C for 2-3 minutes and was put on ice for 1 minute. One microliter of TdT was added to each reaction and incubated at 37°C for 20 minutes. After the incubation, the reaction was inactivated by heating at 65°C for 10 minutes.

PCR of dC-tailed cDNA

A PCR reaction was performed with our GSP2 and the abridged anchor primer (with multi G, provided from the system) to amplify the upstream region of canine *p16* exon 2. The 50 μL PCR reaction contained 5 μL of 10x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of deoxynucleoside triphosphate, 0.4 μM of each primer, 2.5 U of Taq DNA polymerase

and 5 μL of dC-tailed cDNA under mineral oil. The cycling conditions were: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 2 minutes for 35 cycles. The product was analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and detected by UV light.

DNA extraction from agarose Gel

The PCR product from the 5'RACE reaction was extracted from agarose gel using QIAEX II gel extraction kit (Qiaex Inc. Valencia, CA) based on manufacturer's instructions. The DNA band was cut from the agarose gel with a clean scalpel and was placed in a 1.5 mL microcentrifuge tube. The gel piece was weighed on a scale. Three volumes of buffer QX1 to the gel piece were added to the tube and vortexed for 30 seconds. Ten microliters of Qiaex II was added to the sample. The sample was then incubated at 50°C for 10 minutes and was vortexed every 2 minutes. After the incubation, the sample was centrifuged for 30 seconds and the supernatant was removed. The pellet remained in the tube was resuspended in 500 μL of buffer QX1 by vortexing, centrifuged for 30 seconds, and the supernatant was removed. Then the pellet was washed twice with 500 μL of buffer PE. After the final removal of the supernatant, the pellet was air dried for 10-15 minutes and redissolved in 20 μL of sterile, distilled H_2O. The sample was vortexed, incubated at room temperature for 5 minutes, centrifuged for 30 seconds, and the clear supernatant was transferred to a fresh tube for use.

Sequencing

Sequencing reactions were performed by using thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, OH). For each sample, four reactions were carried out. Each reaction mixture contained 2 μL of dGTP master mix, 0.5 μL of

[α-^{33}P]dd GTP, [α-^{33}P]dd ATP, [α-^{33}P]dd TTP or [α-^{33}P]dd CTP, 0.5 μL of reaction buffer, 2.5 μL of DNA, 0.5 pmol of primer and 2 U of thermo sequenase™ DNA polymerase in 7 μL of final volume under light mineral oil. Sequencing reactions were cycled 40 times at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Four microliters of stop solution was added to each tube after the last cycle. Samples were heated at 70°C for 2-3 minutes before loading to the sequencing gel. The sequencing gel contained 6% acrylamide (19:1 acrylamide: bis) and 7 M of urea in 1x TBE with 10% ammonium persulfate and TEMED as catalysts and was run in a vertical sequencing gel electrophoresis apparatus (Bethesda Research Laboratory, Life Technology Inc. Model S2). After the electrophoresis, the sequencing gel was transferred to a chromatography paper (Whatman, Florham Park, NJ) and dried by a gel dryer for two hours. Then a Kodak Biomax MR film (Kodak, Rochester, NY) was exposed to the radioactive gel for 3 days and developed.

Immunohistochemical staining for p16 and p53

Tissue sections of canine nasal carcinomas were used for immunohistochemical evaluation of the expression of p16 and p53 protein. Deparaffinization, antigen retrieval and immunostaining of formalin-fixed paraffin embedded tissues were performed on automated immunostainers. Immunohistochemical staining for p16 was performed on the Bond maXTM Automated Staining System (Vision BioSystemsTM) using the BondTM Polymer Detection System (Vision BioSystemsTM) and a mouse monoclonal antibody against p16 (clone 6H12, NovoCastraTM) at a dilution of 1:20. Antigen retrieval was achieved using the Bond Epitope Retrieval Solution 2 (Vision BioSystemsTM) for 20 min. The immunoreaction was visualized with 3,3-diaminobenzidine substrate (Vision

BioSystemsTM) and sections were counterstained with haematoxylin. Immunohistochemical staining for p53 was performed on the Bench Mark Automated Staining System (Ventana Medical Systems, Inc.) using the Enhanced V-Red Detection (Alk. Phos. Red) Detection System (Ventana Medical Systems, Inc.) and a rabbit polyclonal antibody against p53 (Signet Laboratories) at a dilution of 1:100. Antigen retrieval was achieved using the Ventana Medical Systems Retrieval Solution CC1 (Ventana Medical Systems) for 60 min. Sections were counterstained with haematoxylin. Positive immunohistochemical controls included a canine soft tissue sarcoma with strong p53 expression and normal canine nasal turbinates and lymphoid tissue to which the appropriate antisera were added. For negative controls the primary antibodies were replaced with homologous non-immune sera. Only nuclear staining was evaluated as positive staining for p16 and p53. Nasal carcinomas were divided into positive or negative staining tissues based on the absence or expression of p16 and p53, respectively. Neoplasms with rare single positive staining cells were considered negative for statistical evaluation.

CHAPTER 3

RESULTS

Investigation of viral presence

Forty-three tumor cases including 23 adenocarcinomas, 7 transitional carcinomas, and 13 squamous cell carcinomas were investigated by using PCR with universal herpes primers and the primers for BHV-4. Positive control for universal herpes primers had a predicted 207 bp band on a 2% agarose gel, but no viral amplicons were amplified from the 43 tumor samples. Positive control for BHV-4 had a predicted 153 bp band on a 2% agarose gel, but no viral amplicons were detected from the 43 samples, either (Figure 4).

LOH on canine chromosomal region orthologous to human Ch4p15.1-q12

The polymorphisms of both SNP markers in normal dog population were investigated in 19 DNA samples from normal dogs, including 9 of mixed breed and 9 huskies. For *cCNCG1*, a 301 bp fragment was amplified after PCR. A T at position 266 (in the corresponding GenBank entry) of the gene gave an extra XmnI digestion site. Therefore, samples of 266T homozygous DNA would give three fragments of different sizes (55 bp, 172 bp and 74 bp) after digestion by restriction enzyme XmnI. If there was a C at position 266, one XmnI digestion site would exist to give two fragments of 227 bp and 74 bp sizes. Heterozygous samples with one allele of 266T and one allele of 266C would display four fragments of 227 bp, 172 bp, 74 bp and 55 bp after XmnI digestion. A 148 bp fragment representing *SCGB* was amplified from sample DNA by PCR. An A/G variant at position 132 (in the corresponding GenBank entry) gave a HinfI digestion site on the reverse primer, resulting in one 128 bp fragment and one 20 bp fragment after HinfI digestion. Seven of 19 and 6 of 19 normal samples were heterozygous for the

cCNCG1 and *SCGB* markers, respectively. This showed that polymorphisms could be observed for these two SNP markers. Thirty cases with pairs of normal and neoplastic tissue were then studied for LOH with both SNP markers. For *cCNCG1*, 12 of the 30 cases studied were heterozygous in the normal tissue and thus informative for LOH studies. However, no LOH was found among them. Sixteen of the 30 cases studied were informative for *SCGB*, but no tumor sample showed LOH compared to normal samples. In conclusion, 19 of the 30 cases studied were informative either for SNP marker *cCNCG1* or for *SCGB* on canine chromosomal region orthologous to human Ch 4p15.1-q12, but no LOH was found in these 19 informative samples (Figure 5).

LOH around Canine *p16* region

PCR products of 159 bp, 248 bp and 244 bp were amplified for TTTC, CCA, and CAAAA repeats around the canine *p16* region, respectively. For (TTTC)n, 8 cases were informative, and 2 tumor samples among them showed LOH (Figure 6.1). For (CCA)n, 15 cases were informative but no LOH was found (Figure 6.2). Only few samples showed polymorphisms on (CAAAA)n, so this locus was not studied further. In conclusion, 19 of the 30 cases studied were informative for either (TTTC)n or (CCA)n, and two cases showed LOH for (TTTC)n.

Identification of canine *p16* exon 1 by 5'RACE

The last step of the 5'RACE system is a PCR reaction amplifying the dC-tailed cDNA with one gene specific primer and the abridged anchor primer, which has multiple G to pair with the dC-tail of the cDNA. From this last PCR of 5'RACE, some very faint PCR products were detected on the 2% agarose gel stained with ethidium bromide under UV light. The same PCR was performed again but with gradient annealing temperatures from

54°C to 62°C, increasing 2°C each reaction, to try to optimize amplification. From this repeat PCR reaction, multiple bands were consistently observed on the 2% agarose gel from the five reactions with different annealing temperatures. A clear band of reasonable size (around 400 bp) was purified from the gel and sequenced. Unfortunately, the result of sequencing was unreadable due to too many noises. The sequence will be identified by cloning in the future; this part of the experiments is ongoing.

IHC staining for p16 and p53

The results of IHC staining for p16 and p53 expression in the nucleus were summarized in Table 4. Figure 7 shows the tumor tissue with different levels of p16 and p53 staining. Among the three histopathological types of tumors, squamous cell carcinomas showed most reduction of p16 expression, 72.73% of which had negative p16 staining; followed by adenocarcinomas, 46.15% of which had negative p16 staining. Most of the three types of tumors had negative p53 staining. Positive p53 staining happened most in the cases of adenocarcinomas, which accounted for 38.46% of all adenocarcinoma cases.

CHAPTER 4

DISCUSSION

Canine nasal carcinomas are locally aggressive and typically have poor prognosis. As described previously, very few studies investigated the tumorigenesis of this malignancy and the etiology remains poorly understood. The current study investigated the etiology of canine nasal carcinomas from genetic and virological aspects.

A herpesvirus was first found in association with cancer in the renal adenocarcinomas in leopard frogs in 1934.[31] Other herpesviruses were then found to contribute to the tumorigenesis of several animal and human cancers including Burkitt's lymphoma, Marek's disease, Kaposi's sarcoma and NPC. In this study, the universal herpes primers used can amplify 21 types of herpesviruses (8 human and 13 animal viruses) including EBV and canine herpesvirus; PCR for detection of BHV-4 was also performed because BHV-4 is shown to replicate in many animal hosts including dogs. Among 43 tumor cases we investigated, no viral amplicons were amplified from the samples. According to this result, a significant contribution of herpesvirus to canine nasal carcinomas can be ruled out.

Linkage to the human Ch 4p15.1-q12 region was demonstrated in association with NPC in certain families from Guangdong province in China.[11] We planned to study this region in young onset cases of canine nasal carcinomas, but unfortunately only two cases, one 4 year old and the other 5year old, can be considered "young onset" in the 30 cases we studied. LOH was found in neither of the two young onset cases, nor in the other 28 cases, which were of middle to advanced age. Although these were not all young onset cases and the contribution of this region to the risk of nasal carcinomas in dogs cannot be

completely excluded, LOH at the canine equivalent to human Ch 4p15.1-q12 involvement would seem very rare. Another study in human NPC conducted in another patient population found a susceptibility locus on Ch3p21.31-21.2,[44] which is consistent with the demonstration that deletion of Ch3p is a frequent genetic change in NPC.[16, 27] But this study did not identify any linkage to Ch 4p15.1-q12 nor 9p21. The divergent results between different studies may be due to the different patient populations studied. Considering canine nasal carcinomas, if more young onset cases can be obtained for study, the relevance of the human 4p15.1-q12 orthologous region and onset of canine nasal carcinomas can be addressed more definitely. We can also investigate the region orthologous to human Ch3p21.31-21.2, which might possibly harbor a susceptibility gene.

As an important tumor suppressor gene, inactivation of *p16* has been found in many human cancers.[16,23,26] Lack of p16 expression either at the mRNA or protein level was also reported in tumors including melanomas and osteosarcomas in dogs.[18,22] In our LOH study, 2 of 19 informative cases showed losses with either microsatellite marker around *p16* region in tumor samples. The percentage is 10.53%. The other marker, (CAAAA)n repeat, is located closer to canine *p16* gene than the (TTTC)n repeat and (CCA)n repeat. However, when testing the (CAAAA)n repeat in regular control dog DNA from our lab, it did not show much heterozygosity. Although the ratio of loss in our tumor samples was not very high, it demonstrates the occurrence of loss of *p16* in canine nasal carcinomas. The results of IHC staining confirmed reduced p16 expression, revealing that 72.73% of squamous cell carcinomas and 46.15% of adenocarcinomas lacked p16 protein expression.

The low frequency of LOH around *p16* suggested that *p16* inactivation might be occurring through other mechanisms. Promoter hypermethylation is another important mechanism, which has been reported in NPC.[25] Future study will focus on the identification of the *p16* promoter region, using cloning to identify the correct sequence of the product from 5'RACE, and performing bisulfite conversion reactions and sequencing to investigate the methylation status of the *p16* promoter region in canine nasal carcinomas.

Detection of overexpression of p53 protein in nasal adenocarcinomas by IHC was reported previously.[12] According to this paper, 11 of 19 (57.89%) cases of canine nasal adenocarcinomas showed more than 10% stained nuclei for p53, which indicated the possible p53 mutation or defects in p53 degradation. In our study, only 38.46% of cases of adenocarcinomas had positive p53 staining. The percentage of p53 positive cases in transitional carcinomas and squamous cell carcinomas were even lower. The disagreement between our findings and that of Gamblin RM *et al.*, 1997[12] may be due to the different sample population and detection methods. It is also possible that the mutation of *p53* in our samples resulted in the complete loss of p53 protein or in a mutant form that cannot be detected by this IHC protocol.

While herpesvirus and the canine chromosomal region orthologous to human Ch4p15.1-q12 were not shown to play a role in the etiology of canine nasal carcinoma, inactivation of tumor suppressor gene *p16* seems to be associated. Future studies will focus on promoter hypermethylation of the canine *p16* gene and collecting more cases for IHC to clarify the expression of p16 and p53 protein in this tumor. Moreover, since no cell line derived from canine nasal carcinomas is available currently, establishing tumor

cell lines and normal nasal cell culture for relevant study will help elucidate the etiology of canine nasal carcinomas.

APPENDICES

Table 1 Signalments of 43 cases studied

Case No.	Breed	Age	Sex	Tumor Type
2075147	Mixed	11y11m	M	Adenocarcinoma
2127976	Chow chow	8y	M castrated	Adenocarcinoma
2163839	Belgian tervuren	5y1m	M castrated	Adenocarcinoma
2185356	Mixed	10y6m	M castrated	Adenocarcinoma
2211611	Collie	10y	M castrated	Adenocarcinoma
2238389	Bullmastiff	4y	F	Adenocarcinoma
2240298	Mixed	12y6m	F	Adenocarcinoma
2280046	Labrador	12y	M castrated	Adenocarcinoma
2310045	Beagle	6y	M castrated	Adenocarcinoma
2320270	Shetland sheepdog	10y6m	M castrated	Adenocarcinoma
2357329	Mixed	9y	M castrated	Adenocarcinoma
2519203	Mixed	9y	M castrated	Adenocarcinoma
2559269	Shetland sheepdog	8y10m	F spayed	Adenocarcinoma
2559432	Shetland sheepdog	4y	F spayed	Adenocarcinoma
2658427	Mixed	12y	M castrated	Adenocarcinoma
2658428	Mixed	11y9m	M castrated	Adenocarcinoma
2670468	Mixed	13y	F spayed	Adenocarcinoma
2703343	Mixed	6y	NA	Adenocarcinoma
2713913	Mixed	10y	M castrated	Adenocarcinoma
2744741	German shepherd	6y	F spayed	Adenocarcinoma
2764707	Jack Russell terrier	12y9m	F spayed	Adenocarcinoma
2788446	Basset hound	9y6m	M	Adenocarcinoma
2814870	Mixed	15y6m	NA	Adenocarcinoma
2220353	Chow chow	8y	M castrated	SCC
2221587	Mixed	10y	Male	SCC
2294894	Cocker spaniel	10y	M castrated	SCC
2349596	Basset hound	12y	M castrated	SCC
2446947	Shih tzu	12y	F spayed	SCC
2461986	Golden retriever	16y2m	M castrated	SCC

Table continued on next page

Table 1 continued

2513346	Golden retriever	11y	M castrated	SCC
2555434	Mixed	12y	M castrated	SCC
2709556	Chow chow	9y6m	M castrated	SCC
2759984	Labrador retriever	6y7m	F	SCC
2764845	Labrador retriever	8y	M castrated	SCC
2811976	Labrador retriever	9y	M castrated	SCC
2834357	Labrador retriever	9y	NA	SCC
2179157	Mixed	14y10m	M castrated	TC
2198895	German shorthaired pointer	11y	NA	TC
2542573	Brittany spaniel	13y	M castrated	TC
2609483	NA	7y	F spayed	TC
2685932	Airedale terrier	11y	F spayed	TC
2840551	German shepherd	9y10m	F spayed	TC
2854419	Golden retriever	8y11m	F spayed	TC

M=Male F=Female
SCC=Squamous cell carcinoma
TC=Transitional carcinoma

Table 2 Species of herpesviruses (21) detected by PCR with universal herpes primers

Virus	Common Name	Subfamily	Strain
Aotine herpesvirus 1	Herpesvirus aotus type 1	β	S43E
Ateline herpesvirus 2	Herpesvirus ateles	γ	810
Callitrichine herpesvirus 1	Herpesvirus sanguinus	γ	S-388D
Canid herpesvirus 1	Canine herpesvirus	α	D004
Cercopithecine herpesvirus 5	African green monkey cytomegalovirus	β	CSG
Equid herpesvirus 2	Equine cytomegalovirus	γ	82-A
Feline herpesvirus 1	Feline herpesvirus 1, Feline rhinotracheitis virus	α	C-27
Gallid herpesvirus 1	Infectious laryngotracheitis virus	α	N-71851
Gallid herpesvirus 3	Marek's disease herpesvirus type 2	α	GA5
Human herpesvirus 1	HSV-1	α	MacIntyre
Human herpesvirus 2	HSV-2	α	G
Human herpesvirus 3	VZV	α	Ellen
Human herpesvirus 4	EBV	γ	B95-8
Human herpesvirus 5	Human CMV	β	AD169
Human herpesvirus 6	HHV-6B	β	Z-29
Human herpesvirus 7	Human herpesvirus 7	β	SA
Human herpesvirus 8	Kaposi's sarcoma-associated virus	γ	
Leporid herpesvirus 2	Rabbit herpesvirus; Herpesvirus cuniculi	γ	923J
Psittacid herpesvirus 1	Parrot herpesvirus	α	RSL-1
Saimirine herpesvirus 1	Herpes platyrrhinae	α	MV-5-4
Saimirine herpesvirus 2	Herpesvirus saimiri	γ	S 295C

Table 3

(1) Primer sequences for PCR reactions for investigation of viral presence and LOH

	Forward/Reverse Primer
Universal Herpes	F-TGTAACTCGGTGTAYGGNTTYACNGGNGT R-CACAGAGTCCGTRTCNCCRTADAT
BHV-4	1-CATGACACACTATTTTAAGTACTA 2-ATCTTTCTTTCAGTCTCACTGTA 3-ATAAATTTGTGAAGACAATGGGTA
CNCG-1	F-CACAAACACCCTTGCTGGTC R-GACAACCATATTCCCCTCAC
SCGB	F- ATGTTCTTGATGAATTTTGGC R- AACTGCATACCAATGTGACT
(TTTC)n	F-CTGGCAGGCTCAGAGATTCAGAG R-ACCCACTTTGCTGGCGAATTAGA
(CCA)n	F-TACGGTAAGGAGTGAGGGCTGAC R-TAACGCCACCTTAGAAGTCAGTC
(CAAAA)n	F-TTGCCGAACGCTGTGTTCCGTG R-GGGGCCGGGGGAAGGGTC

(2) Cycling conditions for PCR reactions for investigation of viral presence and LOH

	Cycle Condition	Product Size
Universal Herpes	94°C 30s, 46°C 1min, 72°C 1min for 45 cycles	207bp
BHV-4	94°C 1min, 59°C 2min, 72°C 3min for 35 cycles	153bp
CNCG-1	94°C 1min, 54°C 1min, 72°C 1min for 45 cycles	301bp
SCGB	94°C 1min, 54°C 1min, 72°C 1min for 45 cycles	148bp
(TTTC)n	94°C 1min, 62°C 1min, 72°C 1min for 50 cycles	159bp
(CCA)n	94°C 1min, 62°C 1min, 72°C 1min for 50 cycles	248bp
(CAAAA)n	94°C 1min, 60°C 1min, 72°C 1min for 50 cycles	244bp

Table 4

(1) The number of cases and percentage for each tumor type for positive or negative p16 nuclei staining

	Negative	Positive
Adenocarcinoma	6 (46.15%)	7 (53.85%)
Transitional carcinoma	2 (33.33%)	4 (66.67%)
Squamous cell carcinoma	8 (72.73%)	3 (27.27%)

(2) The number of cases and percentage for each tumor type for positive or negative p53 nuclei staining

	Negative	Positive
Adenocarcinoma	8 (61.54%)	5 (38.46%)
Transitional carcinoma	4 (66.67%)	2 (33.33%)
Squamous cell carcinoma	8 (72.73.%)	3 (27.27%)

(3) The number of cases and percentage for each tumor type of different p53 and p16 staining level combination

	p53+ p16+	p53+ p16-	p53- p16+	p53- p16-
Adenocarcinoma	3 (23.08%)	2 (15.38%)	4 (30.77%)	4 (30.77%)
Transitional carcinoma	1 (16.67%)	1 (16.67%)	3 (50%)	1 (16.67%)
Squamous cell carcinoma	2 (18.18%)	1 (9.09%)	1 (9.09%)	7 (63.63%)

Figure 1 Tumor-suppressive pathway of p16. During the cell cycle, the cyclin D and CDK4 complex phosphorylates pRB, which leads to the release of transcriptional factor E2F to promote cell cycle. p16 competes with CDK4 to bind to cyclin D, which leaves pRB unphosphorylated and bound to E2F, resulting in cell cycle arrest at G1 stage.

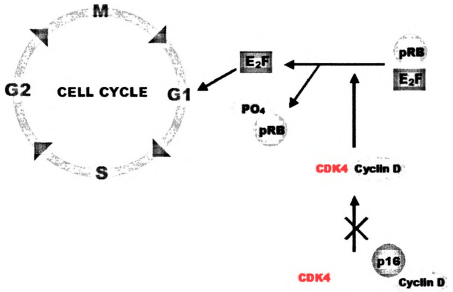


Figure 2 The sequences of intron 3 of *cCNCG1* (X99913) and *SCGB* (AF427093) with SNPs.

The SNPs result in the change of a XmnI and HinfI cutting site. XmnI recognizes the sequence 5'-GAANNNNTTC-3' and cuts between GAANN and NNTTC resulting in a blunt end. HinfI recognizes the sequence 5'-GANTC-3' and cuts between G and ANTC. The reverse primer designed for *SCGB* has one G altered from the correct sequence to create a diagnostic site for SNP. The SNPs are in red and the cutting sites are in gray. The primers designed for PCR are underlined.

>X99913 *cCNCG1*

GTGAGCAGTATGAGCTACTCCTCCATAGCTCTTTTTAAACTCTTATTTAGATG

AAATATAGGTATTCTACTCCTCTATTTAGTGGGCTTGACAAATGCTCCTGCTA

CCCCCACTTCAGAGGATCTTGGGAGTCACTTGCTACTCACCTTTTCCTCTCTTG

GAAAACTGCCACAGCCTGGTGACTATTCATGTCCATGGGGCCTCAAGTCACA

AACACCCTTGCTGGTCATTTTCAGAGGCCTGTCTTTCTTTGTCCCTGAAGAAC

CTCATGCCCCAGTCCCCTATGACTTCTTTCTGACCCTTCTTTCAGGGTCCTGAA

GCAATTGTACGTGGGAGGGGTTGGCTGTGCTCCTCTAAAAAGCTGTGTCCTAT

TTGACCCTACTTTTAGGGAAGTCGAGCACTAGTGTGAGCAGCTAGAAAAGAA

AAGCTAGAAGTCCTTCATAGGAAATGAAACACTCATGAAAACTTCCTACCCG

TGACTCTGAGGGTGTGAGGGGAATATGGTTGTCGGTGTGGATCAGGGGCTCC

CATCCTGTGGAACATATTTACTTTTACTTTGCTTTTGCTCCCTCCCTTTTTCCCC

TCTCTTAATCCTTTTCCCACTGACCACCTTCAGTTTTCCCACTGTTTTTATTTAT

CTTTTCTTCTCTTCCCATCTTCCCTGACAATCTAAGTCTTAGGTCTCTTGCTGG

CAGTTGCAGCATCCAAGGTGGCTCACTTGAGGCCACGGAGGATGCTAAGCTT

GGGTGTTAGCATTGATTAATAGGTTTTTACTAATTACTTTGGTTCATAGCACT

GATACTCAGCAACTCATGGAGGCTCTATATTTTCAAAAATTGTCTAGGCAGTA

GTTAGAACAAAGAGTTTGTTCTTTAAGACTGTTGCTTGCCATGAACATTATAC

AGATGGTGACGATTATGATTTCACTGTACATCTTCATTTTTCAG

C/T **SNP**

>AF427093 ***SCGB***

TTTGTGCAGAGCAGTGTAAGCAAACCAGCATATAAGCCCTCTTTTTAAAGAA

TTCTTCCCAAGTGGATAGAGAGGGGACAGACCAATGCAGTTAAAATTCTTTG

AAGTTAGTCTATTATTTTAAAGTGTTTTTGATAATGTTCTTGATGAATTTTGGC

TGCAGTCTCCTTTGAAAACATACTCATGATAAAGTGTTTTTCTGAAGATTGTC

TTTCAGCAAGGGACAACAAAGCTCAGTGTAGAAAAGAACAAAACTTCTATTA

CGAGTGACATTGGTATGCAGTT

G/A **SNP**

G altered base used to create diagnostic restriction site for SNP

Figure 3 Structure of INK4A/ARF locus.

INK4A/ARF locus in human and mouse gives rise to two distinct transcripts: p16 with exon1 α, exon2 and exon3, and p14 with exon1 β, exon2 and exon3. However, these two proteins are translated in alternative reading frames and share no amino acid homology.

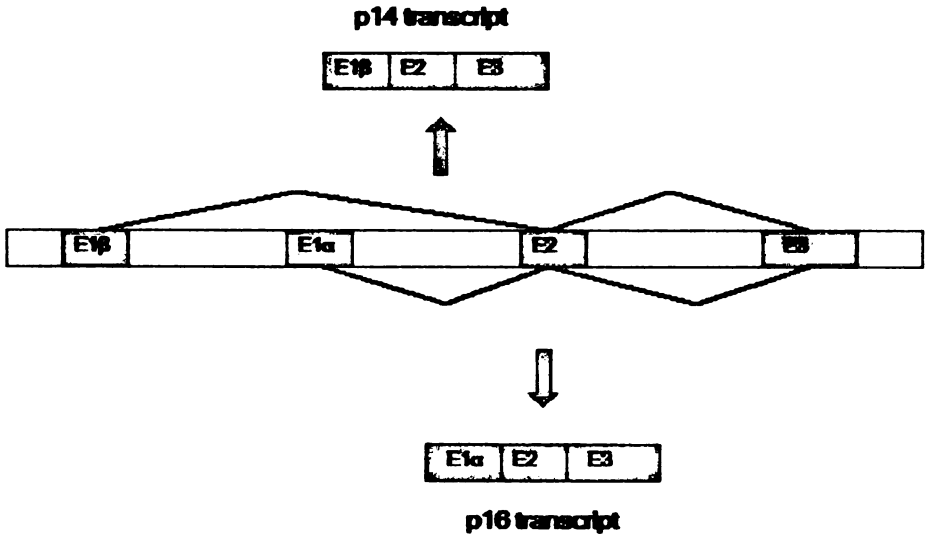
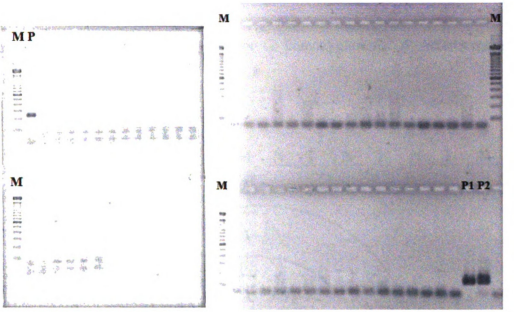


Figure 4 Detection of presence of herpesvirus

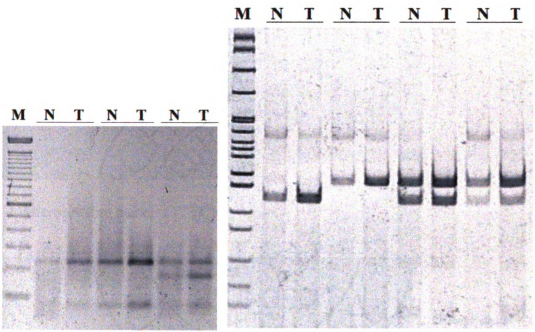


1. Universal herpes primers 2. Primers for BHV-4

P=Positive control, P1=Positive control: FHV-1, P2=Positive control: BHV-4 strain DN599, M=100 bp DNA ladder (Invitrogen, Carlsbad, CA)

Figure 5 LOH study on canine chromosomal region orthologous to human Ch 4p15.1-q12

1. **The first two samples were homozygous for C and the third sample was heterozygous for C T.**
2. From left to right: homozygous for G, homozygous for A, heterozygous for **G A,** heterozygous for G A.

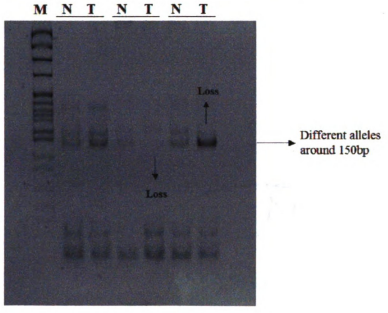


1. SNP marker *cCNCG1* 2. SNP marker *SCGB*

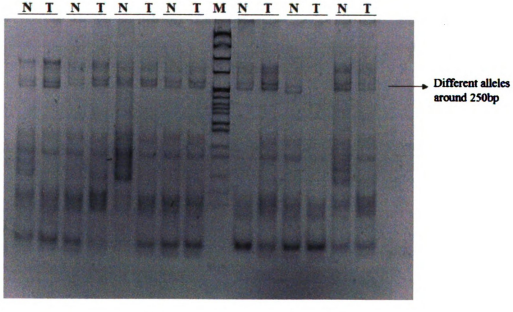
N=normal DNA, T=tumor DNA, M(1)=100 bp DNA ladder (Invitrogen, Carlsbad, **CA),** M(2)=pBR322 DNA-Msp I digest (New England Biolabs, Ipswich, MA) as molecular weight standard

Figure 6 LOH study on canine *p16* with microsatellite markers (TTTC)n and (CCA)n.

The two losses in 8 informative cases for (TTTC)n are shown (1), but no loss was observed in the 15 informative cases for (CCA)n (2).



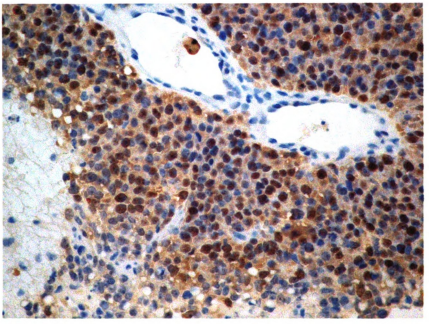
1. Microsatellite marker (TTTC)n



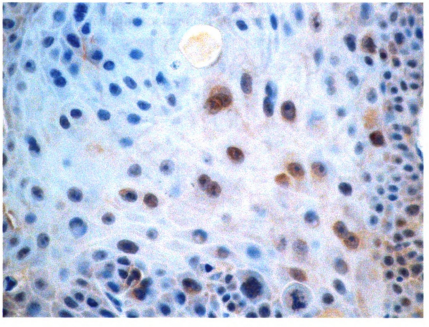
2. Microsatellite marker (CCA)n

M=pBR322 DNA-Msp I digest (New England Biolabs, Ipswich, MA) as molecular weight standard, N=normal DNA, T=tumor DNA

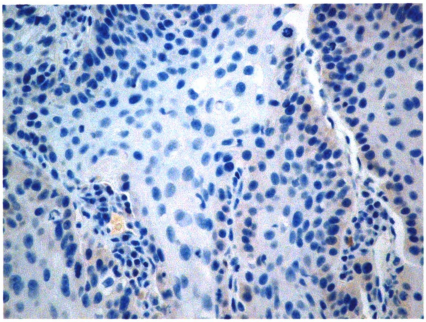
Figure 7a Immunohistochemical staining of canine nasal carcinoma for p16



(1) Nasal carcinoma with high percentage of p16 positive in nuclei

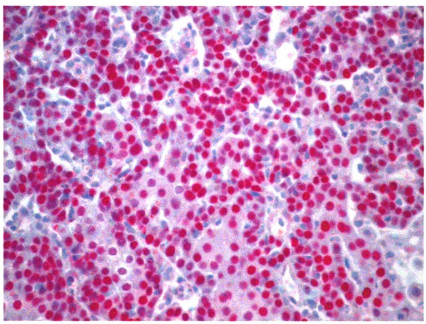


(2) Nasal carcinoma with low percentage of p16 positive nuclei

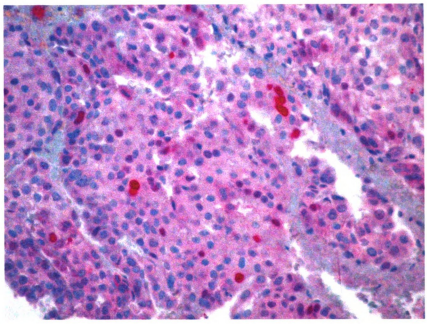


(3) Nasal carcinoma with no p16 staining in nuclei

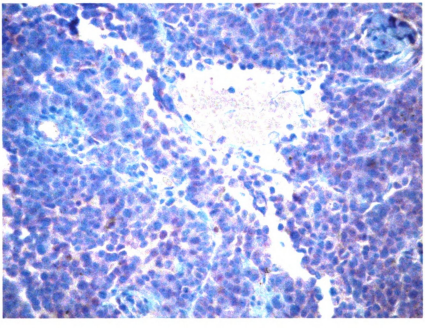
Figure 7b Immunohistochemical staining of canine nasal carcinoma for p53



(1) Nasal carcinoma with high percentage of p53 staining in nuclei



(2) Nasal carcinoma with low percentage of p53 staining in nuclei



(3) Nasal carcinoma with no p53 staining in nuclei

Images in this thesis are presented in color.

REFERENCES

1. Adams WM, Withrow SJ, Walshaw R, Turrell JM, Evans SM, Walker MA, Kurzman ID. 1987 Aug. Radiotherapy of malignant nasal tumors in 67 dogs. J Am Vet Med Assoc. 191(3):311-5

2. Agaoglu FY, Dizdar Y, Dogan O, Alatli C, Ayan I, Savci N, Tas S, Dalay N, Altun M. 2004 Sep-Oct. P53 overexpression in nasopharyngeal carcinoma. In Vivo. 18(5):555-60

3. Banerjee SK, Makdisi WF, Weston AP, Mitchell SM, Campbell DR. 1995 May. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. Biotechniques. 18(5):768-70, 772-3

4. Beck ER, Withrow SJ. 1985 May. Tumors of the canine nasal cavity. Vet Clin North Am Small Anim Pract. 15(3):521-33

5. Brouillette JA, Venta PJ. 2002 Dec. Within-breed heterozygosity of canine single nucleotide polymorphisms identified by across-breed comparison. Anim Genet. 33(6):464-7

6. Bukowski JA, Wartenberg D, Goldschmidt M. 1998 Aug. Environmental causes for sinonasal cancers in pet dogs, and their usefulness as sentinels of indoor cancer risk. J Toxicol Environ Health A. 54(7):579-91

7. Confer AW, DePaoli A. 1978 Jan. Primary neoplasms of the nasal cavity, paranasal sinuses and nasopharynx in the dog. A report of 16 cases from the files of the AFIP. Vet. Pathol. 15(1):18-30

8. Chakrani F, Armand JP, Lenoir G, Ju LY, Liang JP, May E, May P. 1995 May. Mutations clustered in exon 5 of the p53 gene in primary nasopharyngeal carcinomas from southeastern Asia. Int J Cancer. 61(3):316-20

9. Chow LS, Lo KW, Kwong J, To KF, Tsang KS, Lam CW, Dammann R, Huang DP. 2004 May. RASSF1A is a target tumor suppressor from 3p21.3 in nasopharyngeal carcinoma. Int J Cancer. 109(6): 839-47

10. Dahl AR, Hadley WM, Hahn FF, Benson JM, McClellan RO. 1982 Apr. Cytochrome P-450 dependent monooxygenases in olfactory epithelium of dogs: possible role in tumorigenicity. Science. 216(4541):57-59

11. Feng BJ, Huang W, Shugart YY, Lee MK, Zhang F, Xia JC, Wang HY, Huang TB, Jian SW, Huang P, Feng QS, Huang LX, and 18 others. 2002 Aug. Genome-wide scan

for familial nasopharyngeal carcinoma reveals evidence of linkage to chromosome 4. Nature Genet. 31(4):395-399

12. Gamblin RM, Sagartz JE, Couto CG. 1997 Aug. Overexpression of p53 tumor suppressor protein in spontaneously arising neoplasms of dogs. Am J Vet Res. 58(8):857-63.

13. Goldsmith DB, West TM, Morton R. 2002 Feb. HLA associations with nasopharyngeal carcinoma in Southern Chinese: a meta-analysis. Clin Otolaryngol. 27(1):61-7

14. Goyal SM, Naeem K. 1992. Bovine herpesvirus-4: A review. Vet Bull. 62: 181-210

15. Hayes HM Jr., Wilson GP, Fraumeni JF Jr. 1982 Apr. Carcinoma of the nasal cavity and paranasal sinuses in dogs: descriptive epidemiology. Cornell Vet. 72(2):168-179

16. Huang DP, Lo KW, van Hasselt CA, Woo JK, Choi PH, Leung SF, Cheung ST, Cairns P, Sidransky D, Lee JC. 1994 Aug. A region of homozygous deletion on chromosome 9p21–22 in primary nasopharyngeal carcinoma. Cancer Res. 54(15):4003–6

17. Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH. 1994 Apr. A cell cycle regulator potentially involved in genesis of many tumor types. Science. 264(5157):436-40

18. Koenig A, Bianco SR, Fosmire S, Wojcieszyn J, Modiano JF. 2002 Jul. Expression and significance of p53, rb, p21/waf-1, p16/ink-4a, and PTEN tumor suppressors in canine melanoma. Vet Pathol. 39(4):458-72

19. Komano J, Maruo S, Kurozumi K, Oda T, Takada K. 1999 Dec. Oncogenic role of Epstein-Barr virus-encoded RNAs in Burkitt's lymphoma cell line Akata. J Virol. 73(12):9827-31

20. Kruger JM, Venta PJ, Swenson CL, Syring R, Gibbons-Burgener SN, Richter M, Maes RK. 2000 Nov-Dec. Prevalence of bovine herpesvirus-4 infection in cats in Central Michigan. J Vet Intern Med. 14(6):593-7

21. Kyritsis AP, Zhang B, Zhang W, Xiao M, Takeshima H, Bondy ML, Cunningham JE, Levin VA, Bruner J. 1996 Jan. Mutations of the *p16* gene in gliomas. Oncogene. 12(1):63-67

22. Levine RA, Fleischli MA. 2000 Jan. Inactivation of p53 and retinoblastoma family pathways in canine osteosarcoma cell lines. Vet Pathol. 37(1):54-61

23. Liggett WH Jr, Sidransky D. 1998 Mar. Role of the p16 tumor suppressor gene in cancer. J Clin Oncol. 16(3):1197-206

24. Lo KW, Huang DP. 2002 Dec. Genetic and epigenetic changes in nasopharyngeal carcinoma. Seminars in Cancer Biology. 12(6):451-462

25. Lo, KW, Cheung ST, Leung SF, van Hasselt A, Tsang YS, Mak KF, Chung YF, Woo JK, Lee JC, Huang DP. 1996 Jun. Hypermethylation of the *p16* gene in nasopharyngeal carcinoma. Cancer Res. 56(12):2721–2725

26. Lo KW, Huang DP, Lau KM. P16 gene alterations in nasopharyngeal carcinoma. 1995 May. Cancer Res. 55(10):2039-43

27. Lo KW, Teo PM, Hui AB, To KF, Tsang YS, Chan SY, Mak KF, Lee JC, Huang DP. 2000 Jul. High-resolution allelotype of microdissected primary nasopharyngeal carcinoma. Cancer Res. 60(13):3348-53

28. Madwell BR, Priester WA, Gillette EL, Snyder SP. 1976 Jul. Neoplasms of the nasal passages and paranasal sinuses in domesticated animals as reported by 13 veterinary colleges. Am J Vet Res. 37(7):851-6

29. Munro AJ, Lain S, Lane DP. 2005 Feb. P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer. 92(3):434-44

30. Ogilvie GK, LaRue SM. 1992 Sep. Canine and feline nasal and paranasal sinus tumors. Vet Clin North Am Small Anim Pract. 22(5):1133-44

31. Rafferty KA Jr. 1973 Oct. Herpes viruses and cancer. Sci Am. 229(4):26-33

32. Rocco JW, Sindransky D. 2001 Mar. p16(MTS-1/CDKN2/INK4a) in cancer progression. Exp Cell Res. 264(1):42-55

33. Serrano M. 1997 Nov. The tumor suppressor protein p16INK4a. Exp Cell Res. 237(1):7-13

34. Spano JP, Busson P, Atlan D, Bourhis J, Pignon JP, Esteban C, Armand JP. 2003 Oct. Nasopharyngeal carcinomas: an update. Eur J Cancer. 39(15):2121-35

35. Stone S, Jiang P, Dayananth P, Tavtigian SV, Katcher H, Parry D, Peters G, Kamb A. 1995 Jul. Complex structure and regulation of the P16 (MTS1) locus. Cancer Res. 15;55(14):2988-94

36. Sun Y, Hegamyer G, Cheng YJ, Hildesheim A, Chen JY, Chen IH, Cao Y, Yao KT, Colburn NH. 1992 Jul. An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma. Proc Natl Acad Sci U S A. 89(14):6516-20

37. Thompson MP, Kurzrock R. 2004 Feb. Epstein-Barr virus and cancer. Clin Cancer Res. 10(3):803-21

38. Tsimbouri P, Drotar ME, Coy JL, Wilson JB. 2002 Aug. bcl-xL and RAG genes are induced and the response to IL-2 enhanced in EmuEBNA-1 transgenic mouse lymphocytes. Oncogene. 21(33):5182-7

39. VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, Rose TM. 1996 Jul. Detection and analysis of diverse herpesviral species by consensus primer PCR. J Clin Microbiol. 34(7):1666-71

40. Vasef MA, Ferlito A, Weiss LM. 1997 Apr. Nasopharyngeal carcinoma, with emphasis on its relationship to Epstein-Barr virus. Ann Otol Rhinol Laryngol. 106(4):348-56

41. Veske A, Nilsson SE, Gal A. 1997 Nov. Characterization of canine rod photoreceptor cGMP-gated cation channel alpha-subunit gene and exclusion of its involvement in the hereditary retinal dystrophy of Swedish Briards. Gene. 20;202(1-2):115-9

42. Vogelstein B, Kinzler KW. 1992 Aug. p53 function and dysfunction. Cell. 70(4):523-6

43. Wilson JB, Bell JL, Levine AJ. Expression of Epstein-Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. 1996 Jun. EMBO J. 15(12):3117-26

44. Yu MC, Yuan JM. 2002 Dec. Epidemiology of nasopharyngeal carcinoma. Seminars in Cancer Biology. 12(6):421-9

45. Xiong W, Zeng ZY, Xia JH, Xia K, Shen SR, Li XL, Hu DX, Tan C, Xiang JJ, Zhou J, Deng H, Fan SQ, Li WF, Wang R, Zhou M, Zhu SG, Lu HB, Qian J, Zhang BC, Wang JR, Ma J, Xiao BY, Huang H, Zhang QH, Zhou YH, Luo XM, Zhou HD, Yang YX, Dai HP, Feng GY, Pan Q, Wu LQ, He L, Li GY. 2004 Mar. A susceptibility locus at chromosome 3p21 linked to familial nasopharyngeal carcinoma. Cancer Res. 15;64(6):1972-4.