EPIGENETICS OF CANINE LUNG CANCER DEVELOPMENT

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

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And hereby certify that, in their opinion, it is worthy of acceptance.

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LIST OF ABBREVIATIONS

ALK- Anaplastic Lymphoma Kinase

CDA- Cytidine Deaminase

COBRA- Combined Bisulfite Restriction Analysis

CpG – Cytosine-Phosphate-Guanine

DLX1- Distal-Less Homolog 1

EBC- Exhaled Breath Condensate

EGFR- Epidermal Growth Factor Receptor

ETS- Environmental Tobacco Smoke

FOXB2- Forkhead box B2

H&E- Hematoxylin and Eosin

HOXA9- Homeobox A9

HOXB5- Homeobox B5

IHC- Immunohistochemistry

K-Ras- Kirsten Rat Sarcoma Viral Oncogene Homolog

MSP- Methylation Specific PCR

NSCLC- Non-Small Cell Lung Cancer

OSA- Osteosarcoma

PCR- Polymerase Chain Reaction

PPAR (α,β,γ)- Peroxisome Proliferator Activated Receptor (α,β,γ)

ABSTRACT

Background:

Human lung cancer is a leading cause of cancer related mortality and has significant economic impact. Cigarette smoking and exposure to second-hand smoke are a common cause of lung cancer development. Lung cancer is fairly uncommon in dogs despite sharing similar environments, being exposed to second-hand smoke, and having similar respiratory physiology. Understanding the cause of apparent species protection could result in new prevention and treatment avenues for man and dog. One way of investigating species differences is investigation the methylome and protein expression of common cancer related genes in the dog and comparing those to know findings in man.

We elected to study of the methylation status of a panel of canine genes known to be deregulated in human cancer including CDA, DLX-1-3', DLX1-5', FOXB2, HOXA9, HOXB5, and PPAR-γ. During the investigation of these genes in canine lung cancer, and interesting methylation profile was discovered for PPAR-γ. PPAR-γ is a ligand-dependent transcription factor that plays important roles in cellular proliferation and differentiation. It has been implicated as a tumor suppressor in many solid tumors including human prostate, breast, colon, and lung cancer. Our initial findings of an altered methylome of PPAR-γ prompted further investigation of the tissue distribution of PPAR-γ in normal canine lung, canine lung cancer, and metastatic to lung cancer. We also went on to investigate the role of DNA methylation on control of gene expression. The

protein was studied using immunohistochemistry (IHC) and DNA methylation was studied using combined bisulfite restriction analysis (COBRA), and methylation-specific PCR (MSP).

Results:

PPAR-γ is expressed in all large conducting airways, particularly in goblet cells and bronchial glands, in the canine lung. The protein is also expressed in interstitial macrophages. PPAR-γ is expressed in 33% of canine non-small cell lung cancer (NSCLC) cases and 66% of metastatic osteosarcoma (OSA) cases. There is a significant loss of 5' PPAR-γ methylation from normal lung to primary lung cancer and metastatic OSA ($p=0.0002$), however altered PPAR-γ promoter methylation at the interrogated locus does not appear to be associated with changes in protein expression.

Conclusions:

PPAR-γ protein is expressed in normal canine lung tissue, canine primary lung cancer, and metastatic OSA. Confirmation of PPAR-γ protein expression in tumor-bearing dogs supports the investigation of PPAR-γ agonists in this subset of veterinary patients. These results are the first to describe epigenetic marks and protein localization of PPAR-γ among different lung pathologies in the dog.

CHAPTER 1:

INTRODUCTION

LUNG CANCER IN MAN AND DOG

In the United States, lung cancer is the second leading cause of cancer and the most lethal cancer histology, accounting for 28% and 26% of cancer related deaths in men and women respectively.[1] Among primary lung cancer, adenocarcinoma is the most common cancer subtype internationally, and most cases are secondary to cigarette smoking.[2] Additionally, the economic impact of lung cancer is high in the United States with an estimated cost of $12.1 billion annually.[3] Cigarette smoke also has a large health impact on non-smokers, as complications of second-hand smoke exposure are estimated to result in 1.0% of worldwide mortality, with 21,400 of those deaths due to lung cancer development annually. Due to the high incidence and large economic impact of lung cancer, much effort has been placed on cancer prevention, early detection, and new treatment avenues worldwide.

In contrast, primary lung cancer in the domestic dog is considered uncommon however literature on the subject is outdated and sparse.[4,5] This generalization is supported however by pet insurance claims data, which show that primary lung cancer was not a major cause of cancer related insurance claims, accounting for 15 cases per 100,000 dogs per year in the UK.[6] Studies which have attempted to correlate canine lung cancer with second-hand smoke exposure have shown

weak correlations at best, without evidence for increased risk with increasing number of smokers in the home, number of packs smoked in the household per day, or proportion of time the dog spent in the home.[7] A newer study of 30 Yorkshire terriers exposed to at least 2 years of passive cigarette smoke did not report any cases of primary lung cancer, although dogs may not have been screened thoroughly for this condition.[8] Even in dogs experimentally forced to smoke cigarettes, the development of lung cancer appears rare.[9,10] When dogs do develop primary lung cancer, the predominant histologies are of the non-small cell lung cancer (NSCLC) subtypes, with adenocarcinoma being most common, as in humans.[11]

The low incidence of lung cancer in dogs despite over 1/3 of U.S households sharing their home and environment with the dog, suggests that there is an apparent species protection for lung cancer development following exposure to environmental tobacco smoke (ETS).[12] Elucidation of differences between species, specifically cellular pathways that can be targeted, may shed light on the pathogenesis of disease and lead to new diagnosis and treatment avenues.

ALTERED GENE EXPRESSION IN LUNG CANCER

While smoking is considered the major risk factor for lung cancer development, there are several well recognized genetic mutations implicated in lung cancer development. The most common genetic mutations recognized in human non-small cell lung cancer (NSCLC) include activating mutations in epidermal growth

factor receptor (EGFR) and K-ras, as well as rearrangements in anaplastic lymphoma kinase (ALK). Discovery of these mutations has allowed for the use of targeted therapies such as tyrosine kinase inhibitors and monoclonal antibodies, however these discoveries have not been able to have a significant impact in reducing the lethality of primary lung cancer in people.

These same genetic pathway alterations have also been investigated in canine lung cancer. It has been demonstrated that EGFR expression increases with increasing anthracosis, and that the amount of anthracosis is correlated with percentage of positive lung tumor cells. This suggests that combustible materials, which include cigarette smoke, could contribute to EGFR pathway alterations and lung cancer development in the dog.[13] Additionally, activating point mutations in K-ras are present in approximately 15-25% of NSCLC samples in dogs, which is similar to the percentage of human NSCLC cases with this mutation.[14,15] The mutations in K-ras in the dog however, appear to be more similar to the mutations seen in human non-smokers.[15] A study of canine pulmonary carcinoma also found that increased tumor mRNA expression and receptor phosphorylation of the ALK tyrosine receptor was present compared to normal lung tissue.[16]

Epigenetic alterations in human lung cancer are an exciting new topic with a plethora of new information being discovered daily. The aspect of the epigenetic alterations that make this field of research more intriguing than genetic mutations is that is that epigenetic alterations can be detected years prior to cancer

development, can be detected in non-invasive samples, and methylation can be inhibited or reversed in-vivo via methylation inhibitors. While investigation of the epigenome of human lung cancer is an emerging field of study, very little epigenetic studies have been performed in dogs, and no studies have been performed on the dog lung cancer epigenome to the authors knowledge.

EPIGENTIC CONTRIBUTION TO CANCER DEVELOPMENT

DNA methylation is the covalent addition of a methyl group to a cytosine base that is most often 5' to a guanine, this is termed a CpG dinucleotide. In promoter regions of most genes, there is an increased density of CpG dineuclotides, termed a CpG island.[17] When widespread methylation occurs in CpG islands of promoter regions, it can physically prevent binding of transcription factors, and thereby suppress gene expression.[18] During DNA replication, newly synthesized DNA typically acquires methylation patterns identical to the parent strand, thereby making epigenetic changes heritable.[17]

In embryonic development, methylation is believed to play a role in stem cell differentiation, via regulated control of gene expression, which can be passed to progeny cells. This allows for wide phenotypic variance among cells within a single individual. In cancer, methylation of CpG islands can silence tumor-suppressor genes, resulting in uncontrolled cellular proliferation.

Human studies have demonstrated aberrant DNA hypermethylation in critical genes across almost every common human cancer interrogated, including colon,

lung, breast, prostate, gastric, renal, hepatic, bladder, esophageal, ovarian and lymphoid cancers.[19] With the advent of epigenetic cancer screening, it will be important to identify biomarkers that are sensitive and specific, and ideally that can be therapeutically targeted.

The dog serves as an ideal comparative epigenetic model for lung cancer for several reasons. First, the household dog is intimately associated with the human environment. For small dogs especially, exposure to sidestream smoke (a class A carcinogen) is likely high, as they spend more time in intimate contact with their owners.[8] Second, the amount spent on annual healthcare for dogs is second only to human healthcare, at about $18 billion annually in the US.[20] Third, unlike mice, there is much genetic diversity in the dog, and many common human cancers, including lung cancer, develop spontaneously in the dog.[21,22] Finally, due to the relatively rare development of lung cancer in dogs, discovery of differences in the epigenome and the pathways deregulated between the two species could provide new strategies for prevention and treatment in the human counterpart.

EPIGENETIC ALTERATIONS IN HUMAN LUNG CANCER

Recent investigations of the human lung cancer methylome has revealed many epigenetic alterations in both ETS induced and non-smoke induced pulmonary neoplasms. Specific human research examples relevant to our canine research include: the discovery of hundreds of methylated CpG islands within single lung

tumors using methylated CpG island recovery assays, hypermethylation of the p16INK4a in tobacco smoke induced lung cancers, overall increased methylation index in ever smokers as compared to never smokers, and the finding that 82% of NSCLC had at least one aberrantly agene methylated in a panel of 8 genes investigated.[23–26]

Additionally, it has been found that methylation changes occur in low stage cancers and may be developed for early detection of lung cancer. For example, one study was able to identify eight specific CpG island loci showing highly significant hypermethylation in human lung adenocarcinoma, four of which were significantly methylated in low stage tumor samples.[27] Methylation signatures can also be used to determine high risk patients for early intervention. For example, a study published in 2013 found that hypermethylation of five genes was significantly associated with shorter relapse-free survival in stage I NSCLC, and allowed for early intervention in high risk patients with chemotherapy.[28]

Finally, hypermethylated DNA has been found in cancer patients with a variety of tumors from non-invasive samples including sputum, urine, plasma, and exhaled breath condensate, which may allow for non-invasive, inexpensive detection.[29,30]

INVESTIGATION OF EPIGENTIC ALTERATIONS IN CANINE NON-SMALL CELL LUNG CANCER AND OSTEOSARCOMA

This vast literature on the methylation status of human NSCLC sparked an investigation into canine NSCLC tumors. The genes investigated included a

panel of canine primers previously designed by the mentor's (JNB) laboratory. The panel investigated included CDA, DLX1-3', DLX1-5', FOXB2, HOXA9, HOXB5, and PPAR-γ genes (Table 1), as these have been previously implicated in a variety of human cancers. The canine tissues used for methylation specific PCR were all formalin-fixed paraffin embedded tissues from cases presenting to the Veterinary Teaching Hospital for which tissue was collected and archived in the Veterinary Diagnostic Laboratory. Canine primary lung tumors included those cases for which a diagnosis of NSCLC was made between 2005-2011 (see chapter 2- materials and methods). Additionally, we elected to investigate the methylation status using this same panel of primers on canine metastatic to lung osteosarcoma (OSA), in an effort to identify not just genes that allow for a neoplastic niche within the pulmonary parenchyma or genes associated with neoplastic transformation in general, but genes methylated specifically in NSCLC. Canine osteosarcoma was chosen because it is highly metastatic to the lungs and a common histology for which tumor tissue was available for methylation specific PCR (MSP). Control samples were normal canine lung.

HOXA9, HOXB5, DLX1-3', and DLX1-5' did not show methylation in any samples of any histology, although the positive and negative controls amplified as expected. MSP amplification of CDA resulted in repeatable positive bands in both negative and positive controls. CDA expression was variable in all samples (Figure 1). Infrequent methylation of FOXB2 was identified in control samples and NSCLC, but no cases of hypermethylation were identified in OSA.

Peroxisome proliferator-activated receptor was found to have the most interesting methylation profile when comparing NSCLC, metastatic to lung OSA, and normal control lung (Figure 2). For this reason, PPAR-γ was chosen as the next logical step into the investigation of aberrant methylation in canine NSCLC and was the basis for a submitted publication which is also Chapter 2 of this thesis.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR FUNCTION AND CANCER CONTRIBUTION

Peroxisome proliferator activated receptors (PPAR) belong to the steroid/thyroid/retinoid receptor subfamily, are responsive to natural fatty acids, and play a role in lipid metabolism.[31] There are three members of the PPAR family, which include α, β, and γ. The PPARs are divided into six structural domains. The C domain contains two zinc finger-like motifs and is the DNA binding domain, and the E/F domain is the ligand binding domain.[32] Upon ligand binding, PPARs go on to control transcription of many genes. The initial work with PPAR receptors showed their importance in transcription of genes involved in adipogenesis and lipid metabolism, however more recent work has highlighted the importance of PPARs in inflammation and cell differentiation and survival.[33]

Each PPAR has unique transcriptional targets that are somewhat tissue specific. As the methylation and expression of PPAR-γ was investigated in the research presented here, the role of this receptor family will be the focus herein.

PPAR-γ is expressed predominantly in adipose tissue and immune cells including macrophages.[34] The primary ligands for PPAR-γ are 13-hydroxyoctadecadienoic acid (13(S)-HODE) and 15-hydroxyeicosateraenoic acid (15(S)-HETE).[35] Activation of this receptor is principally thought to inhibit proliferation and down regulate inflammation. The anti-inflammatory effects are in part due to antagonizing NFκβ transcription factors.[36] PPAR-γ's role in carcinogenesis is controversial however. For example, PPAR-γ agonists have been found to prevent certain cancers, including colon, breast, prostate and lung.[33,37] Contrary to these findings, other studies have shown that activation results in tumor development.[38] In human lung cancer specifically, it appears that PPAR-γ activation is defective, and the protein is often overexpressed.[35,39] While the exact mechanisms of PPAR-γ contribution to cancer development has not been elucidated, it is fairly well accepted that PPAR-γ agonists inhibit lung cancer development, which suggest that the PPAR-γ acts as a tumor suppressor and that the pathway is deregulated in lung cancer. Further elucidation of these changes could result advances in both diagnosis and therapy.

CHAPTER 2:

PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR γ PROTEIN EXPRESSION IS ASYMETTRICALLY DISTRIBUTED IN PRIMARY LUNG TOMOR AND METASTATIC TO LUNG OSTEOSARCOMA SAMPLES AND DOES NOT CORRELATE WITH GENE METHYLATION

ABSTRACT

Background:

Peroxisome proliferator activated receptor-γ (PPAR-γ) is a ligand-dependent transcription factor that plays important roles in cellular proliferation and differentiation. It has been implicated as a tumor suppressor in many solid tumors including human prostate, breast, colon, and lung cancer. The objective of this study was to determine the tissue distribution of PPAR-γ in normal canine lung, canine lung cancer, and metastatic to lung cancer, as well as determine the role, if any, of DNA methylation in epigenetic control of gene expression. The protein was studied using immunohistochemistry (IHC) and DNA methylation was studied using combined bisulfite restriction analysis (COBRA), and methylation-specific PCR (MSP).

*Results***:**

PPAR-γ is expressed in all large conducting airways, particularly in goblet cells and bronchial glands, in the canine lung. The protein is also expressed in interstitial macrophages. PPAR-γ is expressed in 33% of canine non-small cell lung cancer (NSCLC) cases and 66% of metastatic osteosarcoma (OSA) cases.

There is a significant loss of 5' PPAR-γ methylation from normal lung to primary lung cancer and metastatic OSA (p=0.0002), however altered PPAR-γ promoter methylation at the interrogated locus does not appear to be associated with changes in protein expression.

Conclusions:

PPAR-γ protein is expressed in normal canine lung tissue, canine primary lung cancer, and metastatic OSA. Confirmation of PPAR-γ protein expression in tumor-bearing dogs supports the investigation of PPAR-γ agonists in this subset of veterinary patients. These results are the first to describe epigenetic marks and protein localization of PPAR-γ among different lung pathologies in the dog.

INTRODUCTION

Peroxisome proliferator activated receptor-γ (PPAR-γ) is one of three members (α, β, γ) of the PPAR nuclear hormone receptor superfamily of ligand-dependent transcription factors. Natural ligands for PPAR-γ include fatty acids and eicosanoids.[40] PPAR-γ expression is prominent in adipocytes and its function is best described in regulating lipid metabolism, but PPAR-γ plays a more general role in cellular proliferation, differentiation, and survival, as well as acting as a negative regulator of inflammation.[36,41] More recently, it has also been implicated as a tumor suppressor gene[37], and appears dysregulated in many human cancers including those of prostate, breast, colon, and lung. Research is rapidly

discovering carcinogenic processes in which PPAR-γ is altered at the epigenetic, genetic and protein levels.[33]

One of the most active areas of research is examining the role of PPAR-γ in lung cancer. Both human and murine studies have demonstrated that up-regulation of PPAR-γ can slow lung tumor development via reduced proliferation, decreased production of inflammatory cytokines, and promotion of a more differentiated phenotype.[42–44] Currently, lung cancer is the most common human cancer in the world and the leading cause of cancer related death.[45] Primary lung cancer (PLC) is divided into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) with NSCLC predominating. Despite many advances in human oncologic surgery and chemotherapy, the 5 year survival rate for NSCLC remains lower than 15%.[45] Due to these dismal survival rates, new approaches to cancer therapy are being investigated, including modulation of PPAR-γ. In fact, several studies have shown that artificial activation of PPAR-γ can inhibit growth of lung cancer cells, primarily through differentiation and apoptosis.[46,47]

The dog has proven repeatedly to be an excellent translational model for many human cancers. The dog shares many similarities with humans in respect to genetic aberrations leading to cancer, development of naturally occurring histologically similar cancers, environmental exposures, and similar responses to treatments including radiation, chemotherapy, and monoclonal antibody immunotherapy.[22,48] Although rare in the dog, the species does serve as a good translational model for NSCLC in that dogs naturally develop NSCLC, are

exposed to similar environmental inhalants, have a similar respiratory system anatomy, and similar size and distribution of primary lung tumors.[49] In addition, some genetic aberrations that occur in human NSCLC have also been documented in the dog including k-ras mutations, and altered expression of proteins associated with chemotherapy resistance.[14,50] And finally, the dog has also been used to demonstrate efficacy of new lung cancer therapies in humans, including use of inhalant chemotherapy.[51,52] There is limited research describing PPAR-γ expression in the dog in health or disease. Information is specifically lacking in the contribution of this gene to carcinogenesis. Alterations in PPAR-γ expression have, however, been implicated in canine testicular tumors and nasal carcinomas.[53,54]

There are no reports of PPAR-γ expression in the canine lung or canine lung cancer. PPAR-γ agonists in the thiazolidinedione class (predominantly rosiglitazone) have been studied preliminarily in the dog, including pharmacokinetics and metabolism of this drug.[55,56] More intriguing is that recent research suggests that the combination of PPAR-γ agonists with platinum based chemotherapy are synergistic in treating NSCLC in vitro for human NSCLC and in vivo in murine models. Additionally, safety of oral rosiglitazone and carboplatin was recently published in a phase I clinical trial for cancer-bearing dogs.[57] Given this information, dogs may serve as an excellent model of naturally occurring NSCLC to study the efficacy and tolerability of combination PPAR-γ agonists and platinum-based drugs for treatment of lung cancer. The protein may also be

important in metastatic cancers like osteosarcoma, but this has not been evaluated in the dog.

Given that dogs develop NSCLC and are a good in vivo model for studying the effects of PPAR-γ agonists, it is critical to understand the expression of this protein and possible epigenetic control in the normal canine lung and canine lung cancer. The objective of this investigation was to identify PPAR-γ expression at the protein and epigenetic level in NSCLC and normal lung. In addition, as some protein alterations are necessary for carcinogenesis, and some are cancer specific, we also evaluated metastatic to lung osteosarcoma, to serve as an aggressive model of metastatic to lung cancer and a cancer control. The protein was studied in these groups through immunohistochemistry (IHC) and promoter methylation was studied using combined bisulfite restriction analysis (COBRA), and methylation-specific PCR (MSP). The hypotheses were that PPAR-γ would act as a tumor suppressor, and therefore have decreased expression with increasing malignancy, and that this would correspond with more complete 5' PPAR-γ methylation.

MATERIALS AND METHODS

Tissue Procurement

The University of Missouri Veterinary Diagnostic Laboratory (VMDL) UVIS database was queried from 2005 to 2011 to identify tissue samples for analysis.

For selection of tumor-bearing cases, the database was searched for dogs with a diagnosis of OSA, metastatic OSA, pulmonary carcinoma, pulmonary adenocarcinoma, or bronchiolar carcinoma. Cases were included if a sample or biopsy of the affected lung tissue was obtained and submitted for diagnosis and archiving. The hematoxylin and eosin-stained (H&E) slides from these cases were reviewed by a single pathologist (DYK) to confirm that lung tissue was present and support the original diagnosis of either metastatic OSA or NSCLC. DNA was extracted from paraffin-embedded lung tissue blocks using a commercially available DNA extraction kit (NucleoSpin Tissue, Machery-Nagel. Düren, Germany).

For normal lung control animals, the database was searched for any dogs for which a necropsy was performed and non-diseased lung tissue was reported in the necropsy report and for which formalin fixed and embedded paraffin blocks were archived. Cases were excluded if a metastatic or primary lung tumor was identified, if the dog had cancer elsewhere that could reasonably be expected to metastasize, or if significant lung disease was reported. Cases were also excluded if significant liver or kidney disease was noted in the medical record. The exclusion of these cases was to prevent detection of changes in the methylation status of DNA due to systemic disease. Significant liver and kidney disease was defined as a diagnosis code entry in the patient record to include inflammatory/infectious conditions (any hepatitis or nephritis), any neoplasia in these organs, or any diagnosis code of significant organ dysfunction or failure

(liver failure, hepatopathy, renal failure, nephropathy). In addition, if these diagnoses were noted in the necropsy report, but not entered as a diagnostic code, the cases were also excluded. Finally, cases with an age <5 years were excluded in an attempt to age match the control cases, and exclude methylation changes with age as a confounder. The slides and tissues were then collected, reviewed, and processed as described above.

For both immunohistochemistry and methylation analysis, when more than one tissue type was present in the archived tissue block, only the lung tissue was collected for analysis by trimming away all non-lung derived tissue. Thirteen cases of metastatic to lung OSA (cases O-1 thru 13), 19 cases of NSCLC (cases P-1 thru19), and 22 control cases were identified (cases C-1 thru 22). From these cases identified for inclusion in the study, 10 cases of OSA, 12 cases of NSCLC, and 16 cases of normal lung had tissue available for pathologist review of the H&E stained slides (Table 2 column 8). From the identified cases, 9 cases of OSA (case 0-6 unavailable), 12 cases of NSCLC, and 16 control lung had adequate samples available for immunohistochemistry. Also from the identified cases, 9 cases of OSA (case O-2 unavailable), 12 cases of NSCLC, and 8 cases (C-1, 6, 7, 15, 17, 18, 19, 21) of control lungs had enough DNA to perform COBRA/MSP.

DNA Extraction and Methylation Analysis

DNA was harvested using NucleoSpin Tissue (Machery-Nagel. Düren, Germany) according to the provided protocols. Briefly, xylene and ethanol was used to remove paraffin-wax from the embedded tissue samples. The cells were lysed and proteins digested with Proteinase K, and ethanol was added to adjust DNA binding conditions. The solution was then centrifuged in a spin column provided with the kit to bind the total DNA. The DNA was washed twice and finally eluted as a highly pure product.

COBRA

The MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) website was used to identify an appropriate primer using as input the canine gene located at the 5' end of chr20:6,210,096-6,210,218 CanFam2.[58] The product size of these primers is 124 bp and contains one TaqaI cut site yielding fragments of 86bp and 38bp. The primers used for COBRA were (5' to 3'): forward, TTTTTAGAAGTGTTTGAATTATTGGG and reverse, AAACAAACTCCATACAAAAAACC. PCR was performed at an annealing temperature of 60°C for 60s, an extension temperature of 72°C for 60s, and a melting temperature of 95°C for 60s, repeating for 36 cycles. The PCR product was purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA) and eluted in 20µL of HyPure water. For TaqaI digestion, 10µL of PCR product was added to 2.5µL of Buffer 2, 1µL of TaqaI, and 11.5µL of HyPure water, and incubated at 60ºC for 4h. A schematic representation of the PPAR-γ gene is shown in Figure 8.

MSP was performed on the lung tissue samples described above using PPAR-γ primers created in the region 5' from the promoter from MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Primers were against bisulfite-treated DNA located at chr20:6,206,804-6,210,823. This region was identified in other, unrelated experiments to be hypermethylated in canine cancers (data not shown). The forward and reverse sequences were AATTGATTTATATTGATAGGTTGGC and TTCCATACTAAAATTTAACACGAC respectively. Using bisulfite treated canine DNA from normal spleen, the conditions for MSP were optimized. The methylated primer was used to amplify the region with an annealing temperature appropriate to the primer design for 30s, an extension temperature of 72°C for 30s, and a melting temperature of 95°C for 15s, repeating for 32 cycles. The PCR products were run on a 1.5% agarose gel with Gel Red. The controls for COBRA and MSP consisted of bisulfite converted DNA from normal canine spleen, and DNA from a normal canine spleen methylated in vitro using SssI, then bisulfite converted (to induce methylation of the sample).

Methylation intensity of the COBRA assay was determined using Image J. Regions of interest were created around the region of the primary band (123 bp) and each cut band (85bp and 39bp). A ratio was calculated with the added intensities of the cut bands (methylated) over the intensity of the primary band (unmethylated). The higher the ratio, the greater the relative proportion of methylation in the sample.

Immunohistochemistry

From the archived tissues of adequate condition, 9 cases of OSA, 12 cases of NSCLC, and 16 control lung samples were processed for PPAR-γ immunohistochemistry evaluation and scoring. For immunohistochemistry, the paraffin blocks of the selected cases were sectioned by 5 μm. Anti-human PPAR-gamma rabbit polyclonal antibody (sc-7196, Santa Cruz Biotechnology, Dallas, TX) served as the primary antibody. Heat-induced antigen retrieval using citrate buffer (0.01M, pH 6.0) and EnVision^{TM}+ system (Dako, Carpinteria, CA) were used. The immunoreactivity was visualized by using Romulin AEC Chromogen (Biocare Medical, Concord, CA) and haematoxylin was used as counterstain. In each case, negative controls were included in which the primary antibody was excluded. For a positive control, fresh canine placenta was used, as this has been previously demonstrated as positive in the dog.[59]

Western Blot

Western blot analysis was performed on fresh canine lung and fresh canine placenta confirmed by H&E as microscopically non-diseased tissue as well as human PC3 cells. Tissue lysate was treated with M-PER mammalian protein extraction reagent (Thermo-Fisher, Rockford, IL) as per manufacturer's instruction. Protein concentration was determined using the Bradford method (Thermo-Fisher,Rockford, IL). Equal amounts of protein (~ 40 μg) were separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The blots were blocked at room temperature

for 1 h using Tris-saline buffer (TBS) containing 0.1% Tween 20 and 10% nonfat milk. The membrane was further incubated with primary antibody for PPAR-γ (sc-7196, Santa Cruz Biotechnology) overnight at 4^{o}C. After washing with TBS, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody and visualized with a chemiluminescence detection kit (Thermo-Fisher, Rockford, IL). The blot was imaged using a Kodak imaging station (Carestream Health, Rochester, NY).

Evaluation of Immunohistochemistry

The slides were reviewed and scored by a pathologist (DYK). Some epithelial cells and goblet cells of bronchi and large bronchioles, some bronchial glands, peribronchiolar interstitial macrophages that often were filled with carbon particles, and tumor cells were positive for PPAR-γ IHC expression. Each structure/cell type above was scored separately. The absence of positive staining in bronchi/bronchioles was scored 0 (-) and presence was scored 1 (+). For the peribronchiolar interstitial macrophages, 0 (-) represented zero to few positive macrophages and 1 (+) represented moderate to high numbers of immunopositive macrophages. For tumor cells 0 (-) represented no positivity and 1 (+) represented mild to intense immunopositivity.

Statistical Analysis

Age and gender comparisons were made using an ANOVA on Ranks. Methylation intensities were compared in categorical variables using a Mann-

Whitney U test. Association between gene methylation and tissue expression were made using an ANOVA on Ranks. P-values less than or equal to 0.05 were considered significant.

RESULTS

Patient Demographics

The median ages of dogs were as follows: 8.3 years for the control group, 11.8 years for the NSCLC group, and 8.4 years for the OSA group. The NSCLC group was statistically older than both the OSA and control groups (p: 0.002 and 0.001 respectively). There was no statistical difference in gender between any of the groups (p= 0.658) (Table 2).

Prior to evaluation, all cases (except P-13) were reviewed by DYK to confirm the necropsy report diagnosis. All cases reported as normal lung in the necropsy report were confirmed as such by pathologist review (16 cases). The same was true of all metastatic OSA (10 cases). The NSCLC cases that could be reviewed (12 cases) were further classified as pulmonary adenocarcinoma: papillary type (2 cases) and solid type (1 cases); bronchioloalveolar carcinoma (5 cases); bronchial gland carcinoma (1 case); adenosquamous carcinoma (1 case); and large-cell carcinoma (2 cases) (Table 2).

COBRA

There was a significant difference in methylated band maximum intensities between groups (p=0.0002). Normal lung tissue had the most complete methylation at the evaluated CG site, with primary lung cancer showing relative loss of methylation, and metastatic to lung osteosarcoma being least methylated (Figure 3).

Bisulfite converted normal canine spleen and Sssi treated and bisulfite converted normal canine spleen were both positive for methylation at this site, suggesting that methylation is expected to be present, even in normal tissues.

Methylation Specific PCR

All but one sample of primary lung cancer (case ID C-7) and all samples of osteosarcoma were amplified by MSP using primers for methylated CG. Normal lung tissue samples also amplified with the methylated MSP primers (Figure 4).

The same spleen tissue was used to investigate other genes using MSP primers in an unrelated study including HOXA9, DLX13, DLX15, HOXB5, and CDA and was not methylated in any case (data not shown).

Western Blot

PPAR-γ protein expression of the appropriate molecular weight of 57 kDa was identified via Western blot in both fresh canine lung and fresh canine placenta (Figure 5). Human PC3 cells were used as a positive control.

Immunohistochemistry

Immunohistochemistry performed on canine placenta (positive control) revealed nuclear staining of trophoblast cells as expected for normal PPAR-γ localization (Figure 6).

The majority of bronchi and large bronchioles had strong PPAR-γ positive cytoplasmic immunoreactivity in the epithelium, particularly of goblet cells, and bronchial glands, though not every sample had a large conducting airway present on the slide. In some cases, only tumor tissue was present on the slide, while in others, both tumor and normal tissue were present. Thirty one percent (31%) of control cases, 66% of OSA, and 100% of NSCLC had a large airway available for evaluation, and in every case this airway was positive (Table 2 and Figure 7A).

Most of the peribronchiolar interstitial macrophages that were markedly swollen with carbon particles were strongly positive. If these macrophages were absent or rare, the cases were scored as 0. The cases containing moderate to high numbers of these macrophages were scored as 1 (Figure 7B), which occurred in 0% of control cases, 33% of OSA, and 42% of NSCLC. Tumor tissue was immunopositive for PPAR-γ in 66% of OSA cases and 33% of NSCLC cases. The tumor tissue was partially stained. The positive stains were observed multifocally within the OSA but often at the periphery in the NSCLC (Table 2 and Figure 7C-D).

There was no association between methylation of the gene in primary lung cancer cases and expression of the protein in the tumor tissue (p=0.497), large airways (p= 0.931), or macrophages (p = 0.931). There was no association between methylation of the gene in metastatic to lung osteosarcoma cases and expression of the protein in the tumor tissue (p=0.429), large airways (p= 1.0), or macrophages (p = 0.571).

DISCUSSION

In general, it is believed that in carcinogenesis there is a shift in the epigenetic profile of cells in which genome wide hypomethylation develops, accompanied by regional hypermethylation, specifically in promoter regions of genes. When hypermethylation occurs in promoter regions of tumor suppressor genes, transcription machinery often is impaired, and therefore gene transcription is inhibited. As PPAR-γ is believed to act as a tumor suppressor in lung cancer development, we hypothesized that PPAR-γ promoter would be hypermethylated in all tumor samples, and that frequency of hypermethylation would be greater in the more biologically aggressive cancer (metastatic OSA) than in primary lung tumors. Our findings are the opposite, in that methylation of PPAR-γ was reduced in primary lung cancer as compared to normal lung, and even further loss of methylation occurred in aggressive metastatic to lung osteosarcoma. The interrogated region was selected based on prior data generated in our laboratory identifying methylation at this locus in canine lymphoma. The methylation previously was identified in spite of a lack of a formal CpG island in the region. It

does not appear that the interrogated CG dinucleotides are active in gene expression in the evaluated tissues (Figure 8). These CG dinucleotides may then act as genomic CGs, as opposed to promoter CGs, and therefore have little impact on gene expression. If acting as genomic CGs, loss of methylation with increasing malignancy would be expected, as was identified here. It is also possible that the queried CGs were too far from the transcription start site to modify gene expression.[60] It is also possible, but less likely, that PPAR-γ is serving as a tumor promoter, instead of a tumor suppressor, and that methylation decreases with increasing malignancy, although these data did not demonstrate such a relationship. It is unclear as to why C-7 would not amplify by MSP, though this was the case with multiple attempts at the experiment. In spite of measurable DNA in the sample, it is most likely that the sample was partially degraded, making it difficult to amplify with MSP primers, or that the amplification is below the visible limit of detection.

Bisulfite treatment of DNA results in conversion of cytosine to uracil, but leaves 5-methylcytosine unaltered. Sssi treatment converts all cytosines to 5-methylcytosine.

In these samples, both bisulfite converted normal canine spleen and Sssi treated and bisulfite converted normal canine spleen were positive for methylation. The most logical explanation for this finding is native hypermethylation at the investigated locus. The methylation status of PPAR-γ in normal canine spleen has not been reported previously, and to the authors knowledge has not been

investigated in normal human spleen. PPAR-γ protein expression is reported to be high in the rat spleen however.[34,36] In unpublished data evaluating the methylation status of many other tumor suppressor genes, DNA from spleen tissue of the same dog did not show hypermethylation. The repeatable nature of the finding at this locus, along with negative tumor tissues, supports its validity.

The immunohistochemistry results of this study are the first to describe PPAR-γ protein localization in normal canine lung and canine lung cancer. Studies of PPAR-γ expression in other species are similar to the findings here. Cytoplasmic PPAR-γ expression was found in the epithelium of large conducting airways[61] and in alveolar macrophages.[62,63] PPAR-γ expression has also been described in alveolar epithelial cells in some studies, but was not identified here.[62,63] Reasons for differences across species could be due to true differences in PPAR-γ expression within the lung, due to differences in tissue processing and therefore antibody binding, or due to differences in sensitivity of detection.

This is the first study to describe immunohistochemical positivity for either metastatic or primary lung tumors in the dog. The incidence of IHC positive cases of NSCLC in dogs (33%) is similar to studies in human NSCLC, which found that 42-45% of cases were positive.[64,65] As in human NSCLC[64,65], the cytoplasmic distribution was also found here. It is unclear why PPAR-γ is primarily cytoplasmic in both human and canine NSCLC, however various other carcinomas have described primarily cytoplasmic PPAR-γ staining.[66,67] One proposed mechanism by which PPAR-γ is trapped in the cytoplasm is nitration,

which inhibits translocation into the nucleus. This has been demonstrated in a macrophage-like cell line[68], although further investigation would be necessary to determine if this is the mechanism at play here. In some human NSCLC studies, it has been found that expression of PPAR-γ correlated with tumor type and grade[64], while in other studies no such correlation existed.[65] Due to the small number of samples in this study, no attempt was made to correlate histologic subtype or tumor grade to PPAR-γ positivity. This would be a next reasonable step for future studies where larger patient numbers could be obtained.

To the authors knowledge, IHC evaluation of PPAR-γ in primary or metastatic OSA has not been described in any species. Studies of human OSA cell lines have shown increased PPAR-γ mRNA message, suggesting that this tumor does express PPAR-γ.[69] It is also known that PPAR-γ is important for osteoblast differentiation[70,71], and there is some suggestion that PPAR-γ agonists could inhibit OSA proliferation and induce apoptosis.[72] From the present findings, it is impossible to speculate on the role that PPAR-γ may play in OSA tumorigenesis, but it does provide the first investigation of PPAR-γ expression in metastatic OSA in the dog. In addition, it would be interesting to compare the primary tumor from OSA samples to the corresponding metastatic pulmonary lesions, however this was not possible with the cases available, as the primary tumors were often removed prior to necropsy, making tissues unavailable for comparison.

There are some limitations to the materials in the present study. An attempt was made during case selection to age-match controls to the tumor groups through

exclusion of cases <5 years of age. This arbitrary age was selected to remove very young dogs from the control group, as methylation of tissues has been shown to increase with age.[73] This method allowed for age-matching between the OSA group and the control group, however the NSCLC group was significantly older. These age differences are, however, consistent with age at diagnosis in other reports, in which the average age at diagnosis of primary lung tumors is 11 years whereas the average age at OSA diagnosis is 7-9 years.[5,74] It is very uncommon for animals in the patient database at or around 11 years of age to be submitted for elective necropsy and have histologically normal lung tissue on necropsy and no other tumor capable of metastasis. This explains why an older group of control animals was not identified. Additionally, the tumor type distribution for primary lung tumors is also consistent with what has previously been reported, with adenocarcinoma and bronchoalveolar carcinoma being the most common primary lung tumors of dogs.[15,75]

There was some sample loss in comparing cases that had adequate tissue for IHC as compared to cases that had adequate tissue for COBRA and MSP. The reason for the loss of available tissue for epigenetic studies is a direct reflection of the type/size of samples used for this study. All tissues in this study were obtained from archived patient tissues. All samples were obtained from a veterinary diagnostic laboratory, and as such were considered a part of the patient medical record, therefore, the majority of the tissue had to be preserved to maintain the integrity of the medical record. For all IHC samples, only a single

5um slice needed to be harvested from the archived block, and did not result in deterioration of the patient sample, so could be performed for most cases identified. For DNA harvest, a maximum of 40um slice of tissue could be trimmed from the tissue before interfering with preservation of the patient medical record. In cases of normal control lung, often only small pieces of lung tissue were included in the paraffin embedded block, and were also often combined with up to five other tissues from the same patient. In these cases, only lung tissue was processed. If adequate DNA could not be harvested after a single extraction, the blocks could not be disrupted further. For tumor cases, often the entire paraffin embedded block consisted of the pathologic sample, so there was a high percentage of cases that both IHC and DNA extraction could be performed due to the large amount of preserved tissue. This is the same reason that not all tissue had a large airway available for evaluation of PPAR-γ expression via immunohistochemistry. In general, with the smaller samples available for normal control lung, it is not surprising that many sections did not have large airways present for evaluation. In future studies, using tissue collected specifically for the designed study could result in a higher percentage of cases that have adequate DNA available for methylation analysis as well as ensure that all types of lung tissue (conducting airways and alveoli) would be available for evaluation by immunohistochemistry.

The most important finding of this study is the demonstration of lung tumor positivity for PPAR-γ in dogs with NSCLC and metastatic OSA. There are

currently no published successful medical treatments for either of these conditions in the dog. Dogs with high tumor stage or lymph node metastasis with primary lung tumors have median survival times of <2 months.[76] Dogs with metastatic OSA have a median survival of <2-3 months, even with treatment.[77] PPAR-γ agonists including rosiglitazone and pioglitazone have been studied in the dog, and therapeutic doses and dose-limiting toxicities are published.[78,79] In addition, a phase I clinical trial of oral rosiglitazone and carboplatin in cancer-bearing dogs showed that this combination was safe.[57] The findings of this study provide rationale to suggest that dogs with primary lung tumor and metastatic osteosarcoma could serve as a population which would be appropriate to treat with PPAR-γ agonists, and that expression of PPAR-γ can be demonstrated in these tumor types.

CONCLUSIONS

The results of this study show that PPAR-γ protein is expressed in normal canine lung tissue and in canine primary and metastatic to lung cancer. This report is the first to demonstrate that the frequency of PPAR-γ protein expression in canine primary lung cancer is similar to the frequency described in human NSCLC. These data supports the use of PPAR-γ agonists in this subset of veterinary patients, and suggest that the dog may serve as an appropriate translational model for the study of PPAR-γ agonist use in lung cancer treatment. There are differences in PPAR-γ methylation among normal lung, primary lung

cancer, and metastatic osteosarcoma in the dog, but these differences do not relate to protein expression levels.

CHAPTER 3:

CONCLUSIONS AND FUTURE DIRECTIONS

The science of epigenetics is flourishing in human cancer, as proteins and pathways deranged due to epigenetic alterations are surfacing at an astounding rate. While changes in the methylome are easy to identify, we are only beginning to understand how changes in the methylome translate into altered cellular pathways, how these pathways result in carcinogenesis, and how to target these pathways to improve treatment and prognosis. To the authors knowledge, this is the first study to explore the canine lung cancer methylome, and to identify PPAR-γ expression in canine lung tissue. While further work is still necessary to understand exactly how PPAR-γ contributes to NSCLC carcinogenesis, and how epigenetic differences between canine and human lung cancer result in different species susceptibility, this work is promising. One of the most important aspects of oncologic research is to be able to differentiate between driver and passenger mutations, and comparative oncology is one way to begin to understand which pathways are integral to carcinogenesis, and which are not. While this research does not answer this question, it does suggest that PPAR-γ methylation is altered in NSCLC, and that these changes are not a property of pulmonary neoplasia in general, as the methylome of metastatic to lung cancer in the form of metastatic osteosarcoma was quite different.

There are many future directions that this research could be taken. Some important information could be elucidated simply by improving on the techniques

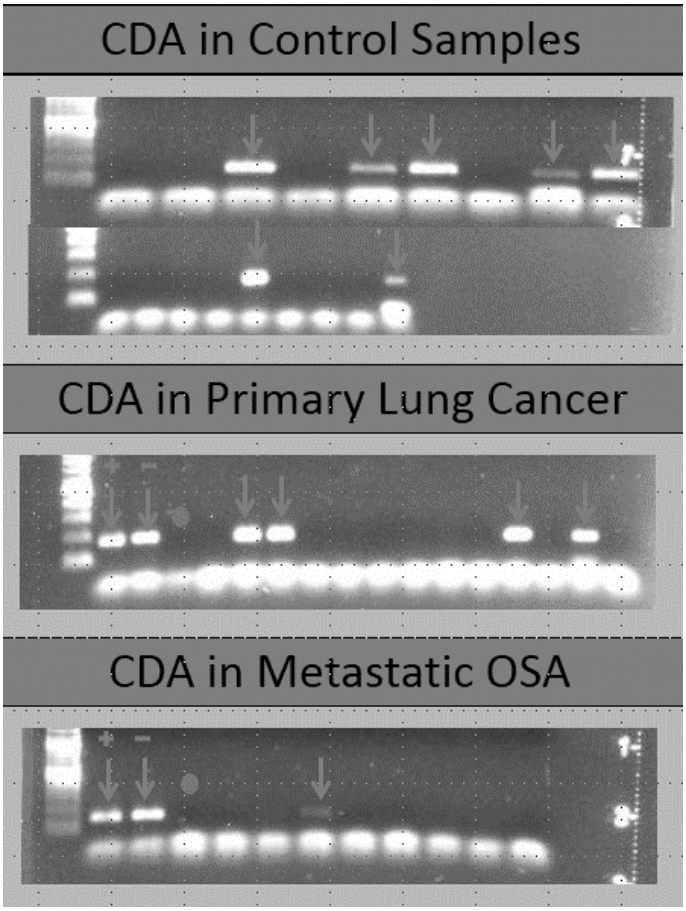
used for this research. The possibility that improved techniques might result in a different outcome are based on our IHC findings. Using IHC we were able to demonstrate PPAR-γ expression on a cell by cell basis. This allowed us to determine that MSP alone was inadequate to make suppositions about how methylation of PPAR-γ contributed to carcinogenesis. This is because MSP from each animal sample included different tissue components dependent upon fresh tissue selection and then processing prior to fixation. It is possible that different MSP results would have been obtained if very precise tissue samples were subjected to MSP, such as just tumor cells or normal pulmonary parenchyma without large epithelium lined airways present. One technique which could achieve this goal is tissue microarray, which would allow for selection of tumor tissue only, and reduce “contamination” from inflammatory cells and epithelium, which appear to also express PPAR-γ. This would allow us to better determine if MSP findings correlated with tumor tissue specifically. Additionally, different tissue processing, such as using fresh tissue and/or pre-defined tissue trimming and processing techniques would have resulted in higher quality DNA for performance of MSP, and may have increased the overall sensitivity of the analysis.

One interesting future direction could be the use of PPAR-γ agonists both in-vitro and in-vivo on canine lung tumors. PPAR-γ agonists that have been experimentally administered to dogs include pioglitazone and rosiglitazone, though mostly in research unrelated to oncology.[55,78,80,81] More recently however,

PPAR-γ agonists have been used in a phase I clinical trial for canine cancer, in which maximally tolerated dose, peak plasma concentrations and the side effect profile of rosiglitazone combined with carboplatin were published.[57] These studies provide some baseline knowledge for PPAR-γ agonist dosing and toxicities so future studies can be performed using this drug in spontaneously arising canine lung cancer. More interesting and promising, is that in-vitro studies investigating platinum combinations with rosiglitazone found that these combinations were synergistic, specifically in NSCLC.[82] Also, a murine study in 2008 found significant regression of drug-resistant lung cancer using this combination, when neither drug alone had efficacy. To the authors knowledge, no reports of combination glitazone therapy with carboplatin has been reported in human lung cancer or in any spontaneous NSCLC model.[83] Further investigation of this combination therapy in dogs may allow for more rapid determination of the benefit of attempting this combination in human NSCLC patients.

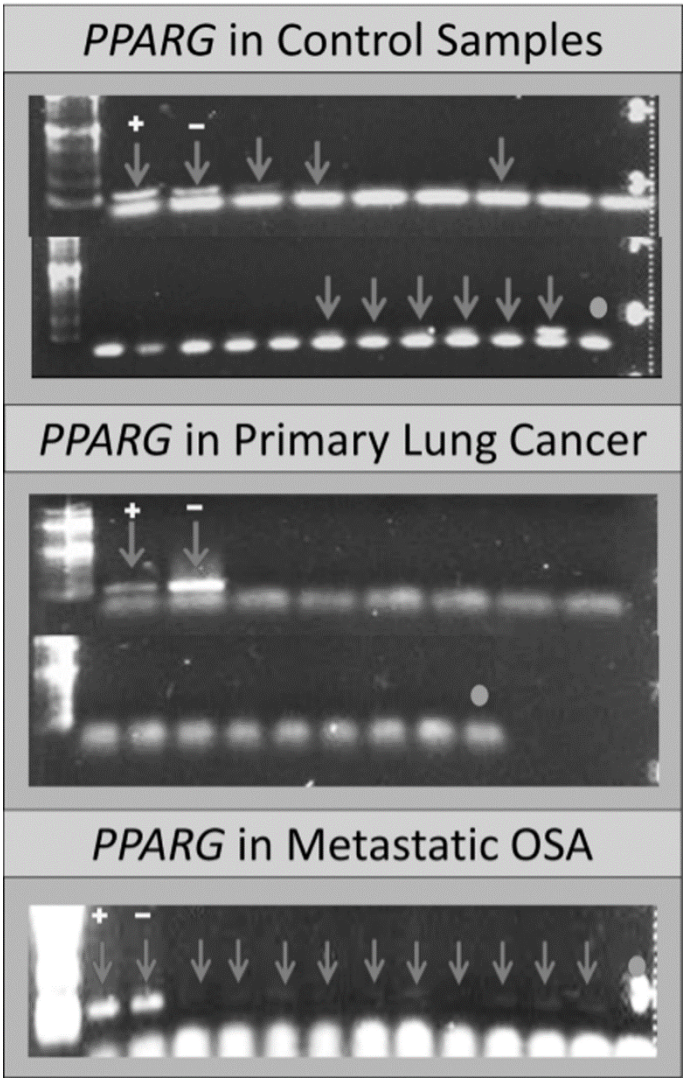
APPENDIX

Figure 1: CDA methylation status in control lung, NSCLC, and metastatic to lung OSA



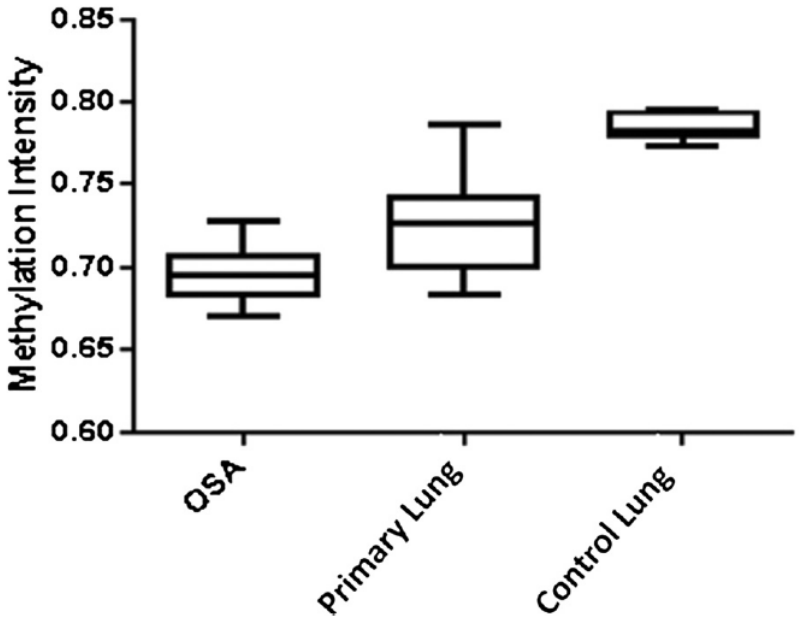
Methylation status of normal control lung (top panel) non-small cell lung cancer (middle panel) and metastatic osteosarcoma (bottom panel). The methylation-specific PCR products were electrophoresed in a 1% agarose gel with Tri-borate containing Gel-Red and visualized with Bio-Doc-UVA Imaging System. (+) methylated in vitro with Sssi and bisulfite converted normal canine spleen positive control (−) bisulfite converted normal canine spleen negative control; grey circle = water template control; arrows = samples positive for methylation. Note that the DNA from dog spleen was methylated in this region in each run without treatment with Sssi and s-adenosyl methionine.

Figure 2: PPAR-γ methylation status in control lung, NSCLC, and metastatic to lung OSA



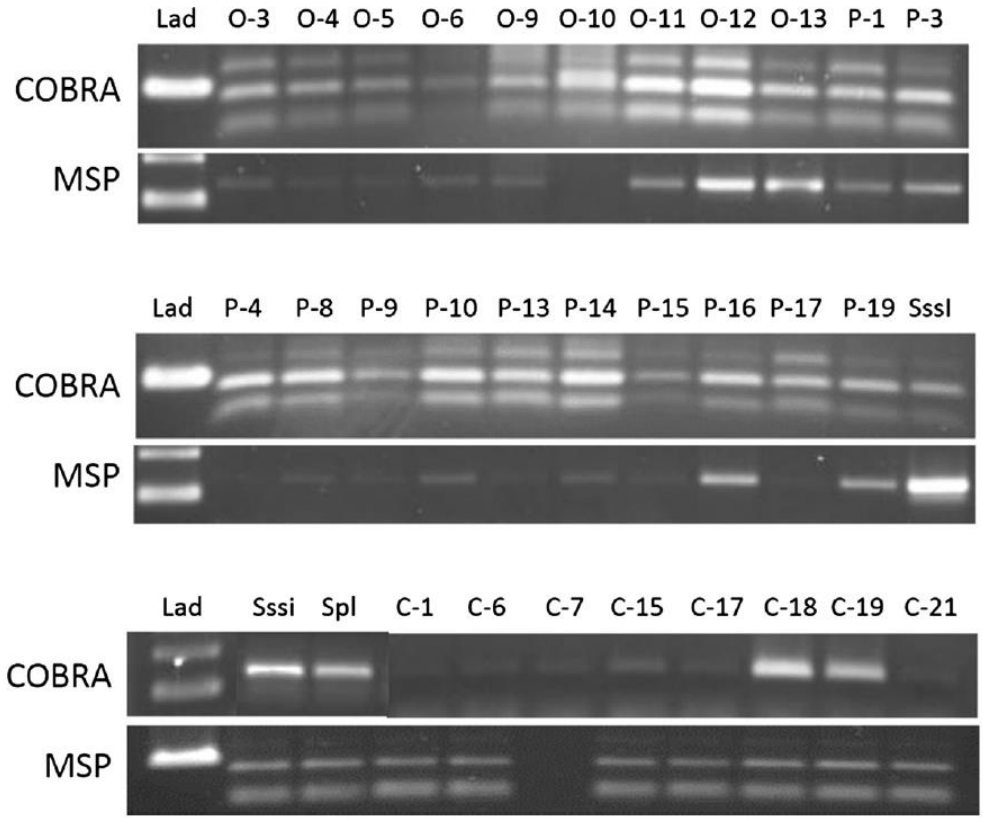
Methylation status of normal control lung (top panel), non-small cell lung cancer (middle panel), and metastatic osteosarcoma (bottom panel). The methylation-specific PCR products were electrophoresed in a 1% agarose gel with Tri-borate containing Gel-Red and visualized with Bio-Doc-UVA Imaging System. (+) methylated in vitro with Sssi and bisulfite converted normal canine spleen positive control (−) bisulfite converted normal canine spleen negative control; grey circle = water template control; arrows = samples positive for methylation. Note that the DNA from dog spleen was methylated in this region in each run without treatment with Sssi and s-adenosyl methionine.

Figure 3: Box Plot of Methylation Intensity of PPAR-γ Osteosarcoma Primary Lung Cancer, and Normal Control Lung.



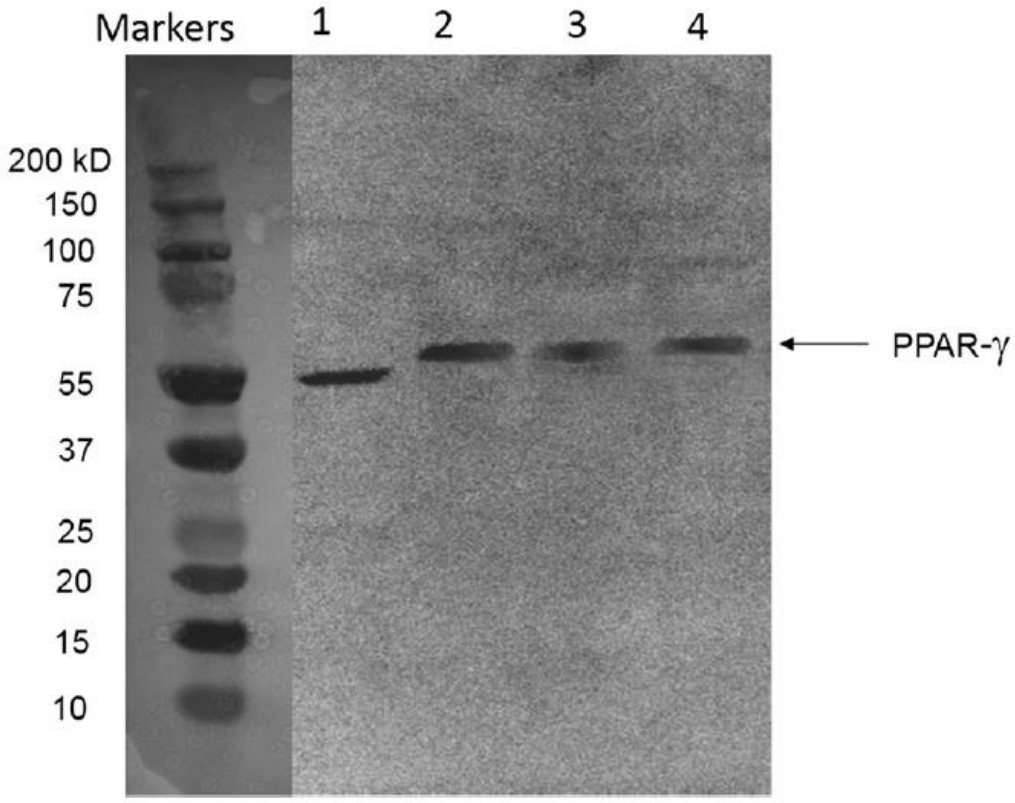
The body incorporates the middle quartiles, the line represents the median values; the whiskers indicate the minimum and maximum values. There is loss of methylation in the primary lung cancer (Primary Lung) as compared to the normal control lung (Control Lung). There is further loss of methylation in osteosarcoma (OSA). The difference is significant (P=0.0002).

Figure 4: Cobra and MSP for Osteosarcoma, Primary Lung Cancer, and Normal Control Lung.



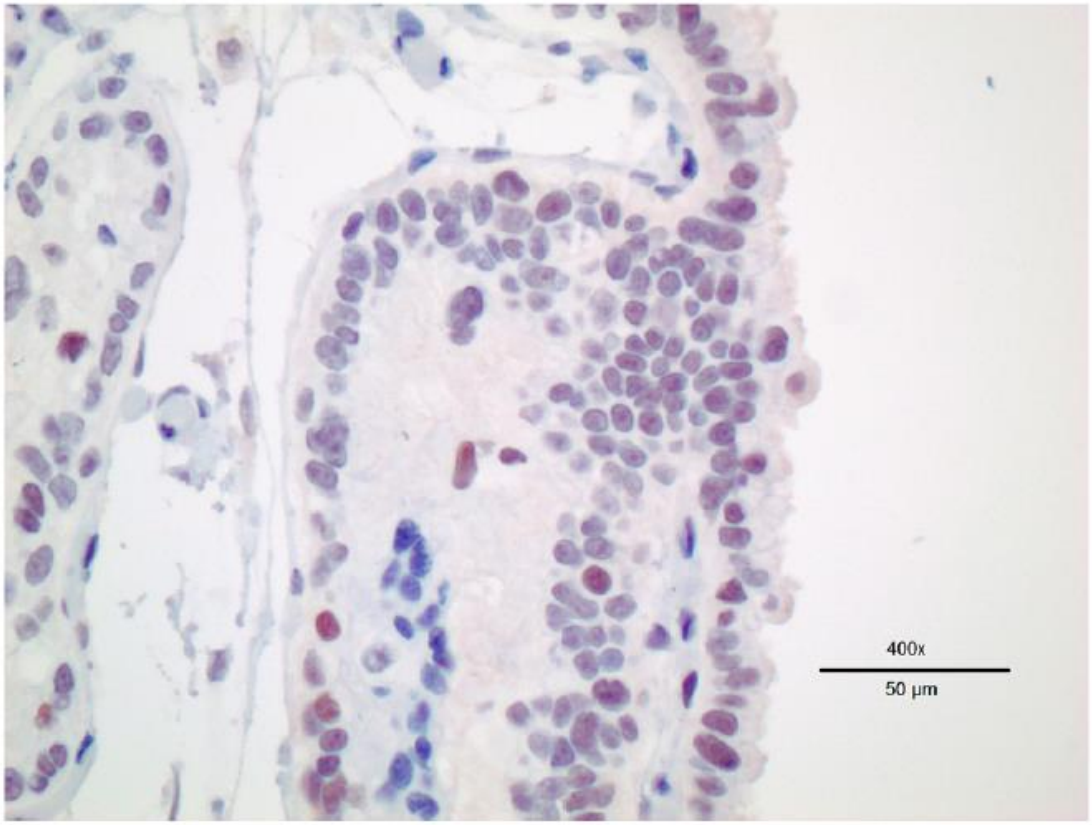
PCR products were run on a 1.5% agarose gel. Lad= 100bp ladder. Samples labeled O-X are osteosarcoma, P-X are primary lung cancer, and C-x are control lung. X represents patient number. Sssi is normal canine spleen DNA methylated in vitro and Spl is normal canine spleen DNA. Sample C-7 did not amplify by MSP.

Figure 5: PPAR-γ Western Blot



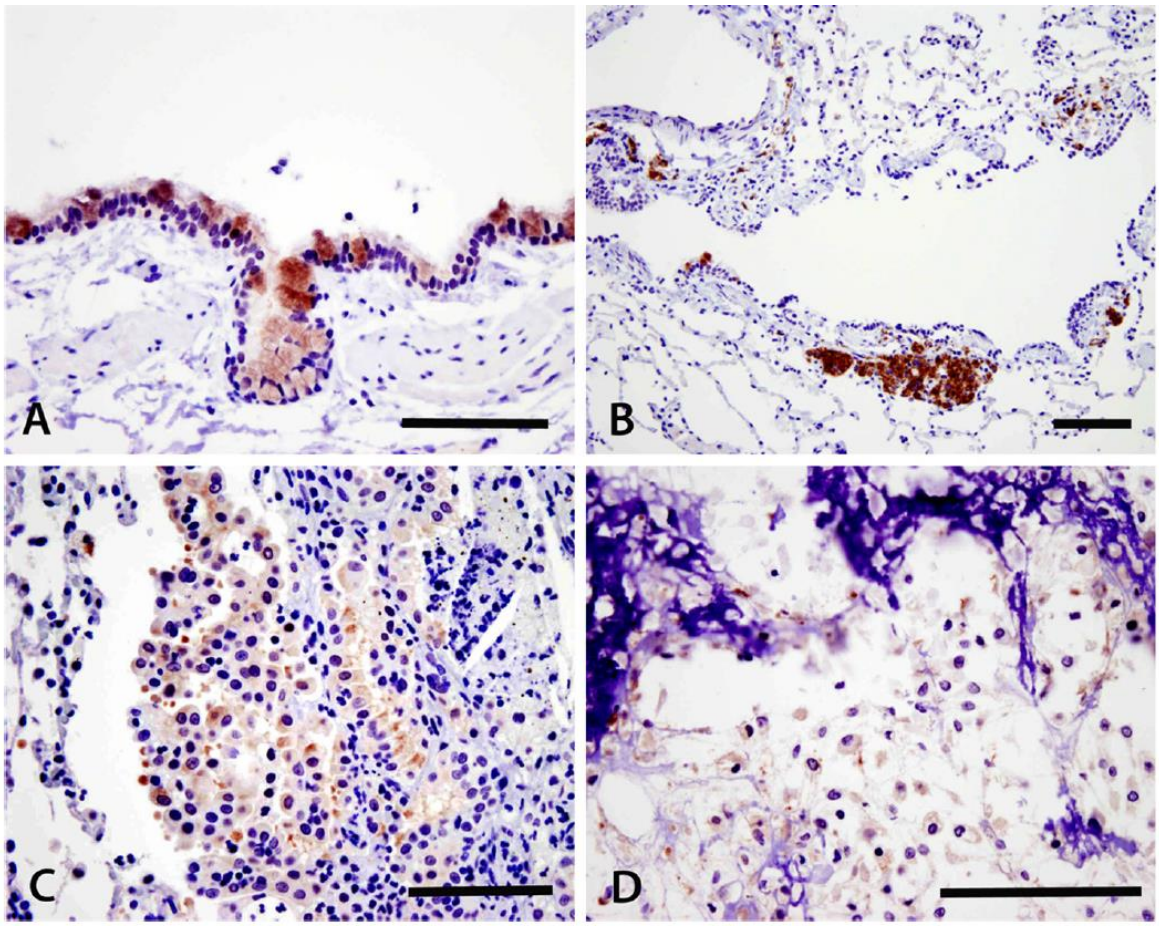
Western blot analysis for the expression of PPAR-γ in fresh samples of human PC3 cells (positive for PPAR-γ) (1), normal canine placenta (2) and two samples of normal canine lung samples (3 and 4) show protein expression of appropriate weight (57 kDa). The predicted canine protein is 38 amino acids longer than the human protein, which is reflected in the decreased migration of the band on Western Blot (human NM-005037 and canine NM_00102463.2). Samples were concurrently formalin fixed and stained with H&E to confirm that they were histologically normal.

Figure 6: Immunohistochemistry of PPAR-γ in Canine Placenta



This 400X view of the canine placenta clearly shows nuclear staining of the trophoblast cells by the antibody

Figure 7: Immunohistochemistry of PPAR-γ in canine lung tissue



The respiratory epithelium of bronchi and large bronchioles exhibited immunoreactivity of PPAR-γ, most strongly in the goblet cells (A) and engorged peribronchiolar interstitial macrophages that were often filled with carbon particles (B). Some of the primary pulmonary adenocarcinomas (C) and metastatic osteosarcomas (D) expressed positive immunoreactivity. BAR= 100µm.

Figure 8: Schematic representation of PPAR-γ gene. The location of the promoter, as well as the sites of interrogation by MSP and COBRA primers is represented

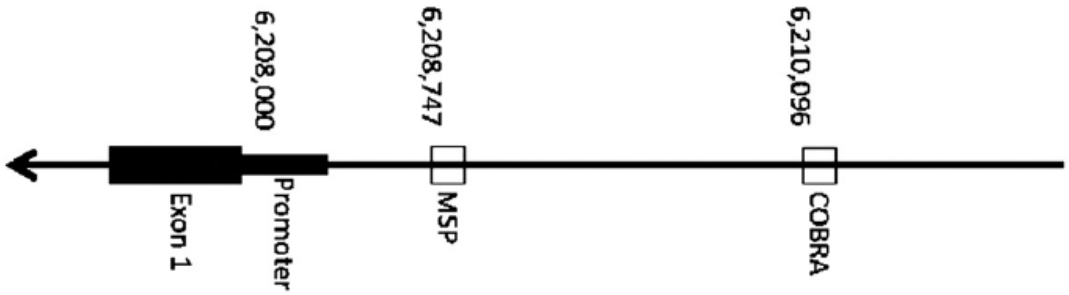


Table 1: Genes for which methylation status was interrogated in canine lung tissue

Gene	Protein Encoded	Function and/or Significance of Protein
CDA25	Cytidine deaminase	Cytidine deaminase enzyme involved in pyrimidine salvaging
DLX1	Homeobox protein DLX-1	Transcriptional regulator
FOXB2	Forkhead box B2	Transcriptional regulator
HOXA9	Homeobox protein Hox-A9	Regulate gene expression, morphogenesis, and differentiation
HOXB5	Homeobox protein Hox-B5	Regulate gene expression, morphogenesis, and differentiation
PPAR-γ	Peroxisome proliferator-activated receptor gamma	Regulates glucose metabolism

Table 2: Signalment and Immunohistochemistry summary for all subjects evaluated

Dog	Age (years)	Sex	Breed	Conducting Airway[b]	Macrophages[b]	Tumor[b]	H&E Histologic Diagnosis
C-1	14	MC	Border Collie	n/a	(–)	n/a	Normal Lung
C-2	4	MC	Australian Shepherd	n/a	(–)	n/a	Normal Lung
C-3	7	MC	Mixed	n/a	(–)	n/a	Normal Lung
C-4	5	FS	Mixed	(+)	(–)	n/a	Normal Lung
C-5	8	MC	Dachshund	(+)	(–)	n/a	Normal Lung
C-6	8	MC	German Shepherd	n/a	(–)	n/a	Normal Lung
C-7	5	FS	Dachshund	n/a	(–)	n/a	Normal Lung
C-9	7	FS	Mixed	n/a	(–)	n/a	Normal Lung
C-10	7	MC	Doberman Pinscher	n/a	(–)	n/a	Normal Lung
C-15	13	MI	Mixed	(+)	(–)	n/a	Normal Lung
C-17	9	FS	Labrador Retriever	(+)	(–)	n/a	Normal Lung
C-18	7	FS	Shih Tzu	n/a	(–)	n/a	Normal Lung
C-19	9	MC	Boxer	n/a	(–)	n/a	Normal Lung
C-20	10	FI	Labrador Retriever	n/a	(–)	n/a	Normal Lung
C-21	12	MC	West Highland Terrier	(+)	(–)	n/a	Normal Lung
C-22	9	MC	Mixed	n/a	(–)	n/a	Normal Lung
O-2	10	MC	Weimaraner	(+)	(–)	(–)	Metastatic Osteosarcoma
O-3	8	MI	Rottweiler	(+)	(+)	(–)	Metastatic Osteosarcoma
O-4	7	MC	Golden Retriever	n/a	(+)	(+)	Metastatic Osteosarcoma
O-5	10	FS	Mixed	n/a	(–)	(+)	Metastatic Osteosarcoma
O-6	5	MC	Labrador Retriever	[a]	[a]	[a]	Metastatic Osteosarcoma
O-9	10	FS	Staffordshire Terrier	(+)	(–)	(+)	Metastatic Osteosarcoma
O-10	8	MC	Great Dane	n/a	(–)	(+)	Metastatic Osteosarcoma
O-11	5	MC	Golden Retriever	(+)	(–)	(+)	Metastatic Osteosarcoma
O-12	10	FS	Mixed	(+)	(+)	(–)	Metastatic Osteosarcoma
O-13	11	FS	Mixed	(+)	(–)	(+)	Metastatic Osteosarcoma
P-1	11	MC	Mixed	(+)	(–)	(–)	Large cell carcinoma
P-3	14	FS	Bichon Frise	(+)	(+)	(+)	Bronchioloalveolar carcinoma
P-4	11	FS	Greyhound	(–)	(+)	(–)	Adenosquamous carcinoma
P-8	9	FS	Scottish Terrier	(+)	(–)	(–)	Bronchioloalveolar carcinoma
P-9	13	MC	Mixed	(+)	(+)	(–)	Bronchioloalveolar carcinoma
P-10	10	MC	Cocker Spaniel	(+)	(–)	(–)	Bronchioloalveolar carcinoma
P-12	10	FS	Boston Terrier	(+)	(–)	(+)	Bronchial gland carcinoma
P-13	10	MC	Boxer	[a]	[a]	[a]	*Acinar Pulmonary adenocarcinoma*[a]
P-14	12	FS	Gordon Setter	(+)	(+)	(–)	Papillary adenocarcinoma
P-15	14	MC	Mixed	(+)	(–)	(+)	Papillary adenocarcinoma
P-16	14	FS	Welsh Corgi	(+)	(–)	(–)	Large cell carcinoma
P-17	15	MC	Welsh Corgi	(+)	(+)	(+)	Solid adenocarcinoma
P-19	11	FS	Mixed	(+)	(–)	(–)	Bronchioloalveolar carcinoma

MC male castrated; *MI* male intact; *FS* female spayed; *FI* female intact; (-) Negative/None/Few/Low; (+) Positive/Mild/Moderate/High/Intense; *n/a* not evaluable/not present

[a]not included in IHC analysis, *italics* = this diagnosis was made by necropsy pathologist, unable to confirm specific subtype, [b]See materials and methods

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