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14-3-3 SIGMA AND P53 IN FELINE MAMMARY CARCINOMA

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EVALUATION OF THE TUMOR SUPPRESSOR GENES 14-3-3 SIGMA AND P53 IN FELINE MAMMARY CARCINOMA

By

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ABSTRACT

EVALUATION OF THE TUMOR SUPPRESSOR GENES 14-3-3 SIGMA AND P53 IN FELINE MAMMARY CARCINOMA

By

Megan Strohmeyer Albertelli

Few studies have examined the genetic changes that occur in feline mammary tumorigenesis. Therefore, we examined feline mammary carcinoma surgical biopsy samples for hypermethylation of 14-3-3 σ (σ) and loss of heterozygosity (LOH) of P53, two common molecular changes found in human primary breast tumors. Feline α was amplified using PCR primers designed for conserved regions in human and mouse α and sequenced. DNA isolated from normal and tumor tissue was modified with bisulfite, amplified by PCR, and sequenced in order to differentiate methylated and unmethylated sites. The number of methylated sites in 5 paired normal and tumor DNA was compared and found to not be significantly different in each case. The number of methylated sites in 8 unpaired samples was compared and no significant difference was found between normal and tumor DNA groups. Therefore, unlike human breast cancer, hypermethylation of α does not play a significant role in feline mammary carcinoma tumorigenesis. P53 LOH was examined by genotyping normal tissue DNA by restriction digest at three single nucleotide polymorphism (SNP) markers in P53. Tumor DNA was then genotyped for cases in which the normal DNA was heterozygous at one or more markers. Of the 20 informative cases studied, 3 cases (15%) showed LOH (complete loss of one allele), 2 cases (10%) showed allelic imbalance (incomplete loss of one allele), 1 case (5%) showed LOH at only one of two informative markers, and 14 cases (70%) showed no LOH. Therefore, the P53 LOH rate is similar between felines and humans.

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CHAPTER 1

INTRODUCTION

1.1 Feline Mammary Neoplasia

Mammary tumors are the third most frequent tumor occurring in the cat (following hematopoietic and skin neoplasia).[1] Mammary tumors make up 12% of all feline malignant tumors and 17% of all tumors in female cats, with an incidence of 12.8 per 100,000 total cats and 25.4 per 100,000 female cats.[1] Intact female cats are at the greatest risk, with ovariectomized females having 0.6 of the relative risk of developing mammary carcinoma and males very rarely affected (less than 1% of total cases).[2] The mean age of occurrence is 10 to 12 years, although cases ranging from 9 months to 23 years have been reported.[3] Siamese cats develop mammary tumors at a younger age (7-9 years) but are not at increased risk after this time period.[2]

Feline mammary tumors are malignant in 80 to 85% of cases, growing rapidly and invasively and metastasizing most commonly to the regional lymph nodes and lungs.[3,4] 80% of these tumors are adenocarcinomas, with tubular adenocarcinomas, papillary adenocarcinomas, and solid carcinomas being the most common types.[3] Feline mammary carcinomas rarely contain estrogen receptors but frequently contain progesterone receptors, and studies have shown that exogenous progestogen administration increases the risk of both malignant and benign mammary tumors.[5]

Surgery is the most common therapy for feline mammary carcinoma. Radical mastectomy (the removal of all mammary glands on an affected side) has been shown to significantly increase the disease-free interval and non-significantly increase survival

time when compared to conservative surgery.[6] Recurrence of the tumor at the tumor site occurs in 66% of cases treated with conservative surgery.[3] Combination chemotherapy with doxorubicin and cyclophosphamide has been shown to induce short-term partial responses (≥50% reduction in tumor mass) in 50% of cats with metastasis or nonresectable tumors, increasing average survival time from 2.5 to 5 months.[7] Adriamycin, mitoxantrone, vincristine, and cisplatin but not recombinant human tumor necrosis factor alpha or recombinant feline interferon gamma have been shown to be effective *in vitro*.[8,9]

Feline mammary carcinomas are generally given a guarded to poor prognosis due to their invasiveness and likelihood of metastasis.[3] The most significant prognostic factor is tumor size, with tumors 1 cm^3 to 8 cm^3 associated with the longest disease-free interval and survival time, while age at diagnosis and breed do not have any prognostic value.[6] There is no standardized histologic grading system that can be related to prognosis for feline mammary carcinomas. The Elston and Ellis method, which grades tumors through assessment of degree of tubule formation, degree of nuclear and cellular pleomorphism, and mitotic count, shows survival time predictive value for well-differentiated carcinomas (grade I) and poorly differentiated carcinomas (grade III) but not for moderately differentiated carcinomas (grade II).[10] The average survival time after detection of a mammary tumor in both treated and untreated cats is 10-12 months.[3] However, cats with tumors of less than 2 cm in diameter have a average survival time of over 3 years, emphasizing the importance of early detection and treatment in this disease.[3] Unfortunately, cats are presented to the veterinarian an average of five months

after the owner initially notes the tumor, and therefore most cats are in an advanced state of disease when treated clinically.[3]

1.2 Cancer Genetics

The etiology of mammary adenocarcinoma is still not fully understood, although environmental toxicants, viruses, and inherited traits have all been suggested as possible causes. These agents may seem diverse, but the common thread between them is that they can damage or propagate changes in normal DNA. All cancers ultimately are caused by the modification or inappropriate activation or inactivation of genes involved in the regulation of cell growth and differentiation, resulting in uncontrolled cellular proliferation. Thus, cancer is a genetic disease.

The process of cellular reproduction, the cell cycle, is normally a tightly controlled process involving many genes that either promote or halt cellular proliferation. The cell cycle consists of four phases: G1 (or gap 1), S (synthesis of DNA), G2 (or gap 2), and M (mitosis, the division of cellular components from the original cell into two daughter cells).[11] While production of cellular proteins in order to double cell size occurs throughout the cell cycle, DNA replication only occurs at a specific point in the process. In order to commit to DNA replication the cell must pass the G1 checkpoint, in which a feedback system assesses cellular and environmental signals for the appropriateness of cell division and triggers the events necessary for synthesis of DNA.[12] After DNA synthesis, the cell must then commit to cell division by passing the G2/M checkpoint, which in a similar fashion to the G1 checkpoint uses a feedback system to assess the state of DNA replication and, when replication is complete, triggers the events necessary for

mitosis.[13] This system ensures that the DNA is undamaged before replication and that DNA replication is completed before mitosis. The feedback system responsible for controlling the cell cycle is based on two families of proteins called cyclins and cyclin-dependent protein kinases (or Cdks). Cdks activate proteins involved in the cell cycle by phosphorylating their serine and threonine residues. Cdks are not active unless bound to cyclins, which are synthesized and degraded in each cell cycle round. Cyclins and Cdks are conserved throughout eukaryotes, although the process is best understood in yeast, where it was shown that a specific Cdk named cdc2 was necessary for advancement through both the G1 and G2/M checkpoints.[11-13] Cdc2 was shown to associate with different cyclins (G1 or mitotic cyclins) in different stages of the cell cycle, which conferred specificity to cdc2 and allowed it to phosphorylate different target proteins at each stage. In mammals, several Cdks and cyclins are involved in passing the G1 and G2/M checkpoints. In order to pass the G1 checkpoint, a mammalian cell requires cdk2,3,4,6 and the cyclin D and E groups.[12] In order to pass the G2/M checkpoint, a mammalian cell requires cdc2 and cyclin groups A and B.[13] See Figure 1.1 for a summary of the cell cycle.

Genetic alterations that predispose an individual to cancer may be inherited or somatic. Inherited, or germline, mutations are passed on from the parents' gametes and are thus found in every cell of the offspring's body. Somatic mutations, on the other hand, occur in individual cells of the body and are not widespread.[14]

These genes are divided into oncogenes and tumor suppressor genes.[14,15] Oncogenes are altered forms of normal cellular genes called proto-oncogenes. Proto-oncogenes encode a number of proteins such as growth factors, growth factor receptors,

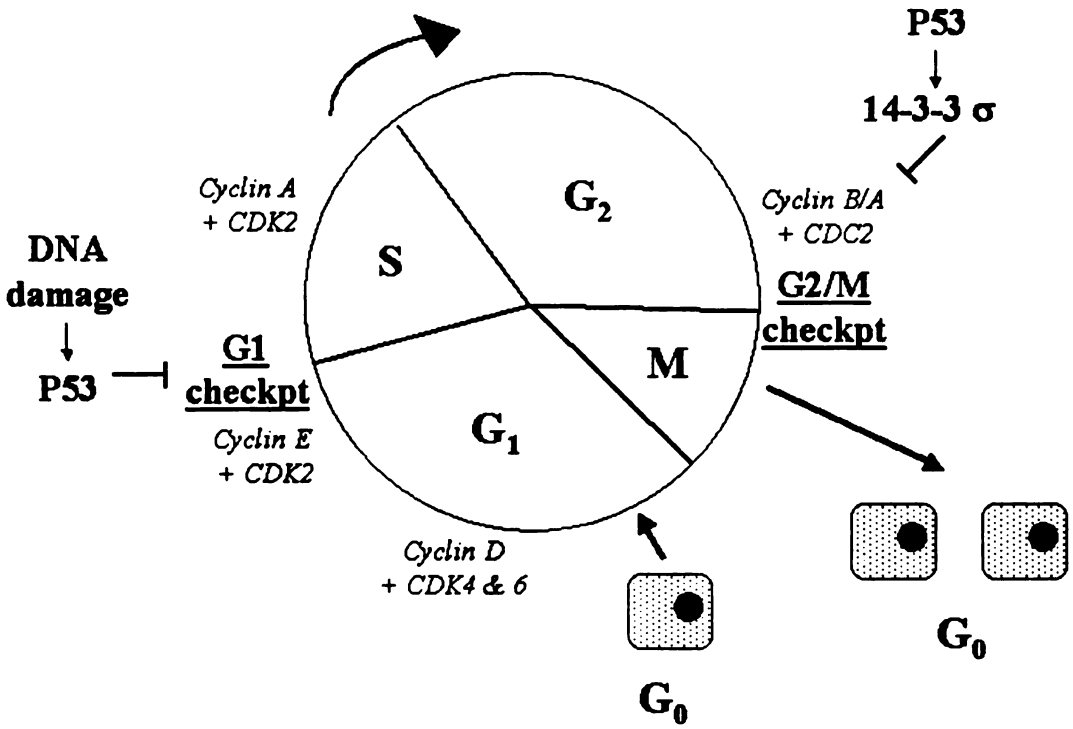

Figure 1.1: The Cell Cycle. Key cyclins and Cdks involved in each phase are shown in italics, checkpoints are shown underlined, and genes studied in this paper are shown in bold. See text for details.

GTPases, and nuclear transcription factors that are involved in tightly controlled signal cascades promoting cellular growth. When these proto-oncogenes escape regulation, cellular growth is continuously supported and cells proliferate without control. Proto-oncogenes can be transformed into oncogenes by a variety of mechanisms. Mutations in the gene, whether deletions, insertions, or base-pair substitutions, can alter portions of the protein product that are important in regulation, resulting in constitutive activity. If the proto-oncogene is near a chromosomal break point, chromosome translocations may place it under the control of a different, more active promoter or

create a constitutively active fusion protein. A proto-oncogene may also undergo amplification, in which a not fully understood mechanism creates multiple tandem copies of the same gene, resulting in increased gene product. RNA viruses may insert an oncogene into a new cell or disrupt the regulation of an existing proto-oncogene. (See recent reviews[14-16] regarding oncogenes.) Alterations in many proto-oncogenes, such as *HER-2/neu*, *ras*, and *myc*, have been observed in human breast adenocarcinomas.[17]

Tumor suppressor genes also regulate the cell cycle but have an antagonistic role to the growth-promoting proto-oncogenes. Since tumor suppressor genes inhibit cellular proliferation, it is the loss of their function that leads to uncontrolled cell growth and neoplasia.[14,15,18] A cell has two copies of each gene and can still function normally providing it has at least one functional copy of a tumor suppressor gene; however, if both copies of a tumor suppressor gene are inactivated then the cell may become tumorigenic. This idea was first developed by Knudson in 1971 and is thus called "Knudson's Two-Hit Hypothesis." Knudson was studying retinoblastoma and noted that familial cases were much more likely to be bilateral and developed at an earlier age when compared to sporadic cases. He proposed that two mutagenic events, or "hits," were necessary to cause retinoblastoma. Sporadic cases were rare as it was very unlikely that two mutational events would occur in the same cell. However, patients with the familial form of the disease had inherited a mutation from a parent and thus had the first "hit" present in every cell of the body. Therefore, the patient would then only need one mutational event to occur in any cell to develop the disease.[19]

Normal tumor suppressor gene function can be lost in a variety of ways. Deletions, insertions, or base-pair substitutions can alter or halt protein expression. The

whole gene may be lost when a portion of a chromosome is deleted or an entire chromosome is lost in non-disjunction during mitosis. An epigenetic process such as hypermethylation, described in more detail in chapter 3, may silence the expression of an otherwise normal gene. As mentioned earlier, a cell can function normally with one good copy of a tumor suppressor gene. However, if the one good gene is lost through any of the methods described above, or replaced with an additional copy of the non-functional gene through such mechanisms as chromosomal non-disjunction and reduplication of the remaining chromosome or mitotic recombination, then the cell may become tumorigenic. One hallmark of a tumor suppressor gene is loss of heterozygosity (LOH), in which normal cells are heterozygous for a genetic marker within or near the tumor suppressor gene of interest. However, tumor cells only contain one allele of the marker, suggesting that the individual harbored one functional copy and one nonfunctional copy of the tumor suppressor gene, and that the tumor cells have lost the functional copy. (See recent reviews[14,15,18] regarding tumor suppressor genes.)

Although the classic tumor suppressor gene model suggests that both copies of the gene need to be inactivated in order for tumorigenesis to occur, recent evidence suggests that haploinsufficiency, or loss of only one functional allele, may contribute to tumorigenesis. This effect may be due to dosage sensitivity of a gene product, or may affect the cell when combined with mutations in other oncogenes or tumor suppressor genes.[20,21] Few examples of haploinsufficiency and tumorigenesis are currently known, but future work in this field may discover new tumor suppressor genes despite the absence of LOH.

1.3 Cats as Animal Models for Human Breast Cancer

Breast cancer is the most common malignancy of women, with one of every eight women in the U.S. likely to develop the disease.[17] There are many similarities between feline mammary tumors and human breast cancers that make cats suitable animal models for therapeutic trials and the study of mammary tumorigenesis. Adenocarcinoma is the predominant type of mammary tumor in both cats and humans.[2] Both species exhibit a high rate of metastasis with the most common sites of metastasis being the regional lymph nodes and the lungs.[8] Women and cats most often develop mammary cancer during middle age[8] and tumor size is an important prognostic indicator in both.[22] Unlike canine mammary tumors, feline mammary tumors are responsive to chemotherapeutic agents used to treat human tumors, particularly doxorubicin.[23] Mammary carcinoma in Siamese cats suggests a genetic component to the disease as their younger mean age of tumor development when compared to other breeds is similar to the earlier onset of breast cancer in women from families with a history of breast cancer.[2] The main difference between feline and human breast cancers is hormone receptor status: 70% of human breast adenocarcinomas are estrogen receptor (ER) positive as compared to 10% of feline mammary adenocarcinomas.[2,22] Therefore, feline mammary tumors may provide an excellent model for ER negative human breast tumors.

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CHAPTER 2

SUMMARY OF CASES

2.1 Description of Sample Population

Feline mammary adenocarcinoma cases for study were randomly selected from surgical biopsy samples submitted to the Animal Health Diagnostic Laboratory (AHDL)[1] from January 1, 1997 to April 30, 2000. 211 total cases were represented. The majority of cats were Domestic Shorthair (177/83.9%) followed by Siamese (18/8.5%), Persian (3/1.4%), Himalayan (1/0.5%), Maine Coon (1/0.5%), Ragdoll (1/0.5%), and Tonkanese (1/0.5%). The breed was not reported for nine cases (4.3%). The median age of the population was 11.3±3.6 years. The majority of cases were female (196/92.4%) with 8 cases (3.8%) being male and 8 cases (3.8%) being unreported. Of the females, 138 of the 196 cases (70%) were ovariohysterectomized and 58 cases (30%) were intact. All males were neutered.

2.2 Tumor Types

Generally, feline mammary adenocarcinomas can be classified based on three patterns of proliferation. Papillary adenocarcinomas arise from the epithelium of mammary ducts and appear as papillary projections. Tubular adenocarcinomas also arise from mammary duct epithelium but form tubules rather than papillary projections. Lobular adenocarcinomas, also called acinar or alveolar adenocarciomas, form distinct acini that may be divided by connective tissue septa into lobules. Often a tumor may contain more than one proliferation pattern. Mammary carcinomas may also be classified

[1] Currently known as the Diagnostic Center for Population and Animal Health

as solid carcinomas, which contain solid nodules or sheets of neoplastic epithelial cells not arranged in one of the patterns described above. Mixed mammary tumors contain neoplastic epithelial cells as well as neoplastic myoepithelial cells with differentiation into cartilage and bone. A mammary carcinoma may be described as scirrhous if it is accompanied by collagenous connective tissue proliferation.

Of the 212 cases, lobular adenocarciomas were the most common (82 or 38.7%) and papillary adenocarcinomas were the second most common (61 or 28.7%). There were no cases classified as strictly tubular adenocarcinomas although three tumors (1.4%) were classified as having both tubular and papillary patterns. A summary of tumor types for all 212 cases can be found in table 2.1.

2.3 DNA Isolation

Feline mammary adenocarcinoma samples were obtained as formalin-fixed paraffin-embedded samples. A veterinary pathologist (Dr. Yamini) examined a section of each sample and outlined the tumor with permanent marker on the corresponding paraffin block, thus demarcating mammary adenocarcinoma from normal mammary tissue. Tissue was then excised from the middle of the indicated tumor region as well as outside the indicated tumor region in order to obtain samples of adenocarcinoma cells and normal cells from each sample.

A small section (3 mm in diameter and 0.5 mm thick) of either tumor or normal tissue was obtained with a scalpel blade and placed in 400 µl of digestion buffer (50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween®). Each sample was then heated at 95°C for 10 minutes, pulsed in microwave twice for 30 seconds at high power, and cooled to room

temperature. Each sample was then digested by adding proteinase K and incubating at 42°C overnight. Each sample was then heated at 95°C for 10 minutes to inactivate the proteinase K and centrifuged at 12,000 rpm for 10 minutes. An aliquot of this digested lysate was used as a template for PCR or then modified with bisulfite treatment to study methylation.

Table 2.1: Tumor Type Summary

Tumor Type #	Cases	Percentage
Lobular Adenocarcinoma	82	38.9
PapillaryAdenocarcinoma	61	28.9
Unspecified Adenocarcinoma	15	7.1
Lobular Adenocarcinoma + Solid Carcinoma	12	5.7
Papillary + Lobular Adenocarcinoma	11	5.2
Solid Carcinoma	9	4.3
Lobular + Scirrhous Adenocarcinoma	5	2.4
Scirrhous Adenocarcinoma	4	1.9
Tubular + Papillary Adenocarcinoma	3	1.4
Papillary Adenocarcinoma + Solid Carcinoma	3	1.4
Mixed Tumor	2	0.9
Papillary + Scirrhous Adenocarcinoma	2	0.9
Scirrhous Adenocarcinoma + Solid Carcinoma	1	0.5
Adenocarcinoma w/ Squamous Differentiation	1	0.5
Totals	211	100

CHAPTER 3

14-3-3 σ

3.1 Background

14-3-3 σ, hereafter called σ, is a member of the highly conserved 14-3-3 gene family, which in mammals consists of at least seven isoforms with the general function of facilitating protein-protein interactions.[1] Human σ is a 9876 bp gene (Genbank accession AF029081) consisting of one 1245 bp exon which produces a 25 kDa protein.[2] The gene is located on human chromosome 1p35.[3] σ was first identified in 1992 and called human mammary epithelial marker 1 (HME1)[2] while another group independently identified the gene in 1993 and called it stratifin.[4]

σ protein is expressed only in epithelial tissues, especially those enriched in stratified squamous keratinizing epithelium.[4] Expression of σ results in arrest of the cell cycle at the G2/M checkpoint.[3] The σ promoter contains a p53 binding site and σ expression is induced by p53, a transcription factor expressed in response to DNA damage.[3] Once expressed, σ protein binds CDK2 and CDC2 in the nucleus, transports them out of the nucleus by means of a nuclear exporting signal and sequesters them in the cytoplasm. CDK2 and CDC2, key proteins in the G2/M checkpoint of the cell cycle, are unable to phosphorylate nuclear proteins from the cytoplasm and thus the cell does not proceed to mitosis.[5] Human colorectal cancer cells deficient in σ initially arrest at the G2/M checkpoint but are unable to maintain this state, undergoing cell death in "mitotic catastrophe" as they enter mitosis.[6] σ deficient cells also show more frequent

chromosomal aberrations (such as chromosomal breaks, end-to-end fusions, and unbalanced translocations) than cells containing σ.[7] Without σ to maintain G2/M arrest, these chromosomal changes go unrepaired, leading to genetic instability and increasing the likelihood that these cells undergo carcinogenesis.

σ was first associated with human cancer in 1992 when the first report describing the gene noted that while normal human mammary epithelial cells expressed σ mRNA, two cell lines from spontaneous human mammary carcinomas expressed greatly reduced amounts of σ mRNA.[2] In 1999, σ was shown to be overexpressed in a mitoxantrone resistant pancreatic adenocarcinoma cell line, leading the authors to suggest that σ modulated protein kinase C which in turn upregulated proteins involved in drug resistance.[8] Ferguson et al then reported in 2000 that σ mRNA expression was undetectable in 94% of primary human breast tumors examined, although loss of heterozygosity or mutations of the gene were extremely rare. However, 91% of primary human breast tumors and breast tumor cell lines examined contained hypermethylated CpG islands in the 5' portion of the σ gene, silencing σ expression (as further explained in the following paragraph).[9] Another study performed proteomic profiling of normal and tumor human breast tissue and found that all primary breast tumors examined contained an average of 10-fold less σ protein than normal breast tissue.[10] Hypermethylation of σ was also identified in human gastric cancer, colorectal cancer, and hepatocellular cancer cell lines and was observed in 43% of primary gastric adenocarcinomas examined.[11]

Many genetic changes have been noted in carcinogenesis but it is a relatively new

observation that epigenetic changes such as hypermethylation also play a role. An epigenetic phenomenon creates heritable states without altering the DNA nucleotide sequence itself. Methylation is an epigenetic event that is part of the regulation of gene expression in normal cells. Methylation occurs at cytosine nucleotides that are 5' to guanine nucleotides in the genome; these sites of potential methylation are called CpGs. CpGs are methylated by DNA methyltransferases (DNMTs). DNMT1, responsible for maintenance of methylation, recognizes hemimethylated CpGs and methylates the unmethylated site. DNMTs responsible for *de novo* methylation have not been identified although DNMT3α and β are strong candidates.[12] CpGs occur at a less then expected frequency in the genome, but some stretches of DNA, called CpG islands, contain the expected or higher than expected frequency of the dinucleotide. CpG islands are often located in the promoter and 5' coding regions of genes and play in a role in regulation of gene expression. Genes with methylated CpG islands are not transcribed; examples are imprinted genes and genes on the inactive X chromosome in females. Genes with unmethylated CpG islands are transcribed; these are often housekeeping genes. The mechanisms of this transcriptional control are not completely understood, but it is known that the chromatin surrounding methylated CpG islands is in a "closed", transcriptionally inactive state characterized by de-acetylated histones. Chromatin surrounding unmethylated CpG islands, on the other hand, is in a transcriptionally favorable state characterized by acetylated histones. Levels of methylation within each CpG island are variable, but even a small amount of methylation results in a significant decrease in gene expression. In one experimental system, methylation of 7% of CpGs within a CpG island resulted in 67-90% reduction in gene expression while higher levels of methylation

silenced the gene completely.[13] For general reviews of methylation, see Momparler and Boveni, 2000;[14] Baylin and Herman, 2000;[15] and Robertson and Jones, 2000.[16]

Methylation can contribute to carcinogenesis in two ways. The first is mutation; methylated CpGs are hotspots for mutation as the 5-methylcytosine may deaminate to form thymine, resulting in a C to T transition mutation. The second is hypermethylation, in which normally unmethylated CpG islands are methylated by an unknown mechanism, thus silencing expression of a normally expressed gene. If this silenced gene is a tumor suppressor, the cell now lacks this protein and may begin to uncontrollably proliferate, resulting in a tumor. Hypermethylation may occur in both copies of a tumor suppressor gene, or may silence one copy while the other is inactivated through mutation or deletion. Hypermethylation of several tumor suppressor genes has been noted in several types of human cancers and demethylating chemotherapeutic agents such as 5-azacytidine and decitabine are currently in clinical trials (see http://www.cancernet.nci.nih.gov).

Hypermethylation has not been studied in the cat. Further characterization of this process is the first step in determining if demethylating chemotherapeutic agents will also be effective in the cat. As hypermethylation of σ is a common event in human breast cancers and feline and human breast cancers share many similarities, I hypothesize that hypermethylation of σ is a frequent event in feline mammary cancers and may represent an important target for intervention.

3.2 Materials and Methods

3.2.1 Sequencing Feline 14-3-3 σ

σ has been sequenced in the human, mouse, and sheep and is highly conserved

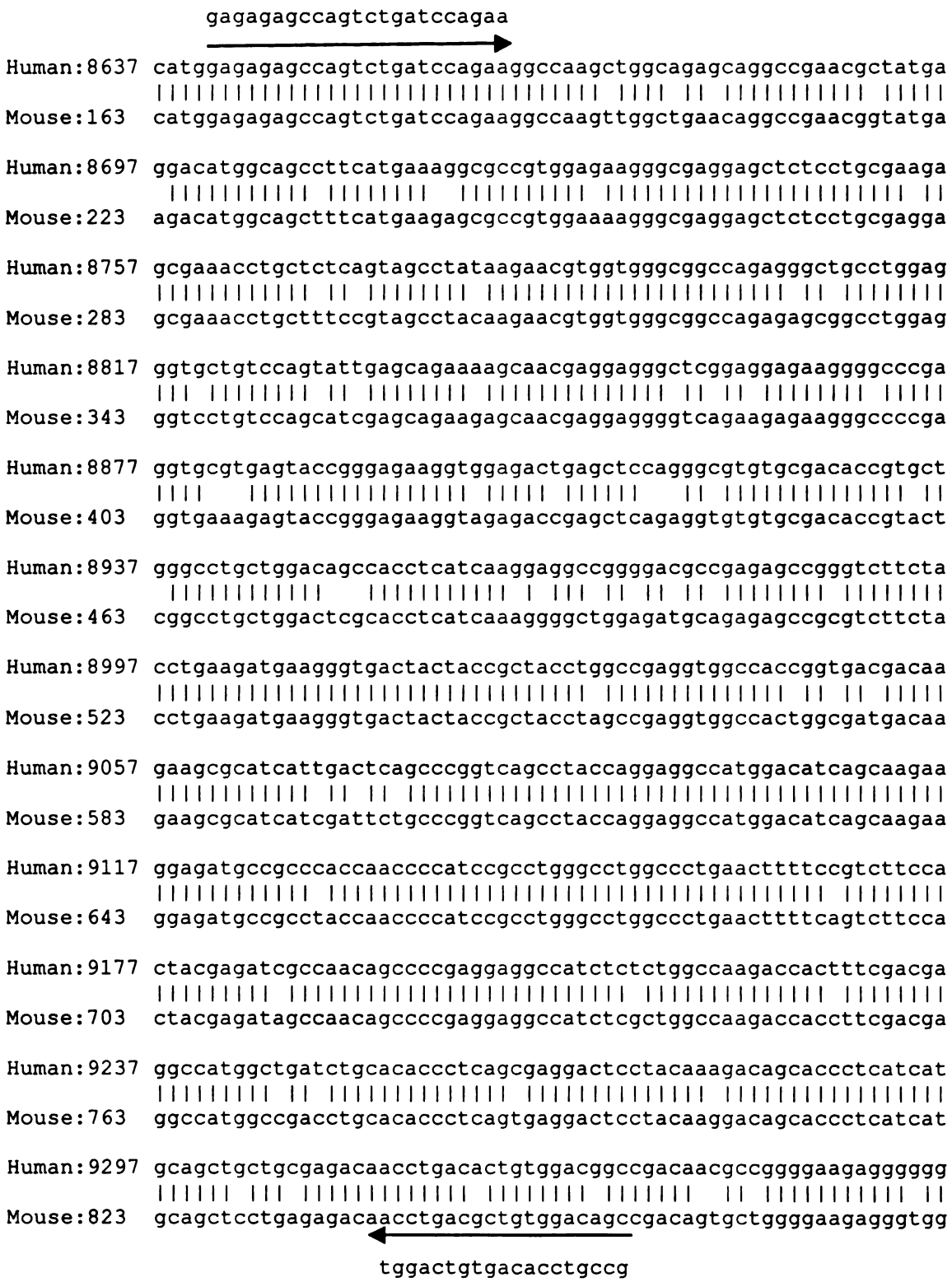
between these species. Figure 3.1 demonstrates the homology between the human and mouse σ sequences, with each vertical line representing a conserved nucleotide.

As feline σ has not been previously sequenced, the most highly conserved regions between the sequenced species were used to design several primer sets with the intention of amplifying σ exon 1 from feline DNA by polymerase chain reaction (PCR). During primer design, care was taken to not place the 3' end of the primer at the 3rd codon position, as this is most likely to be variable, decreasing the likelihood of amplification. These primer sets were then used to amplify both human and feline genomic DNA isolated from white blood cells in order to optimize PCR conditions. Primers used most successfully to amplify σ from the human and the cat are shown in figure 3.1 and were used in a 25 μl PCR reaction containing 2.5 mM $MgCl_2$, 2.5 U Taq, and 0.4 μM each primer. The PCR conditions consisted of 40 cycles of 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes. PCR products were visualized on 1% agarose gels and appropriately sized bands were excised with a clean scalpel blade. DNA was purified using the Qiaex II bead kit (Qiagen) according to the manufacturer's directions. DNA was sequenced on an ABI 377 automated sequencer (Applied Biosystems) using Big Dye terminators (Applied Biosystems). The feline sequence was then examined for CpGs in order to assess the potential of feline σ for methylation.

3.2.2 Methylation Status of Feline Mammary Adenocarcinoma Cases

In order to assess methylation of normal and tumor tissue samples from feline mammary adenocarcinoma cases (as described in Chapter 2), DNA was treated with bisulfite and then sequenced. Bisulfite treatment chemically modifies cytosine nucleotides of DNA to uracil, but does not modify methylated cytosines. During

Figure 3.1: Human and Mouse σ. Vertical lines represent conserved nucleotides. Primers designed for conserved regions are shown. Genbank accession numbers: AF029081 (human), AF058798 (mouse).



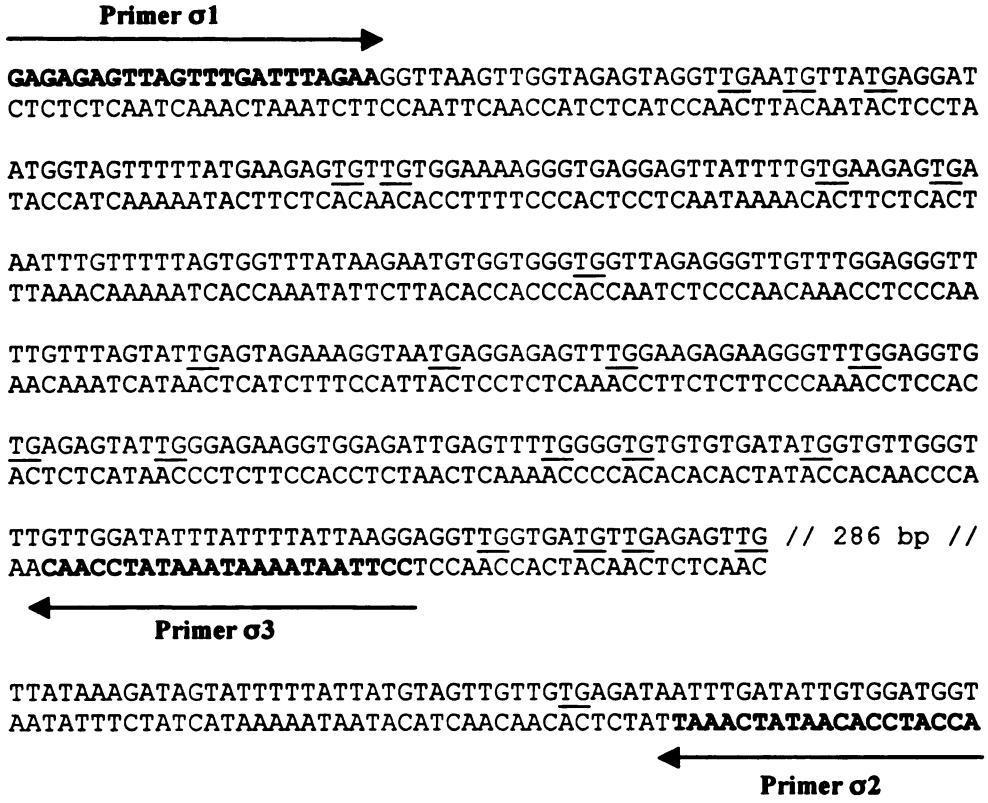
subsequent PCR the uracils are converted to thymine. Samples are then sequenced and the methylation status can be determined by examining each CpG (the locations of which were determined in previous sequencing of an unmodified sample) for the presence of a cytosine or a thymine. Formalin-fixed paraffin-embedded tissue has been shown to be a useful source of DNA for this technique as the fixation does not affect methylation.[17]

In order to perform bisulfite treatment, DNA was isolated from formalin-fixed paraffin-embedded tissue samples as described in chapter 2. Approximately 1 μg of DNA and 20 μg glycogen were incubated in 0.2 M NaOH (50 μl total volume) for 10 minutes at 37°C. 30 μl of freshly prepared 10mM hydroquinone and 520 μl of freshly prepared 3.5 M sodium bisulfite were added to each sample and incubated under mineral oil for 16 hours at 50°C. Samples were purified with Wizard PCR Preps (Promega) and eluted with 50 μl water. Samples were then treated with NaOH (0.3 M final concentration), incubated for 5 minutes at room temperature, and ethanol precipitated.

Once modified, the DNA was amplified with primers designed for the bisulfite-modified sequence. Primers were designed for regions without CpGs in order to eliminate potential sequence variations between methylated and unmethylated DNA that may have interfered with amplification. A heminesting PCR strategy was used to amplify the bisulfite modified DNA as shown in figure 3.2. In the first PCR, 2.5 μl – 5 μl of the modified DNA sample was added to a 25 μl reaction containing 2.5 mM $MgCl_2$, 0.4 μM primer σ1, and 0.4 μM primer σ2. The reaction was boiled for 3 minutes, 0.25U Taq was added, and the reaction was cycled 35 times at 94°C for 1 minute, 54°C for 2 minutes, and 72°C for 3 minutes. 1 μl of this completed PCR was then added to a second 25 μl reaction containing primers σ1 and σ3. All conditions were the same as the first PCR

except the annealing temperature of 56°C. The final amplified product was 325 bp and contained 17 CpG sites.

Figure 3.2: Primers Used in the Heminesting PCR of Bisulfite-Modified σ. Primers are shown in bold and modified CpGs are shown underlined.



Once amplified, the PCR product was visualized on a 1% agarose gel and appropriately sized bands were excised with a clean scalpel blade. The excised DNA was purified with the Qiaex II bead kit (Qiagen) according to the manufacturer's directions. DNA was then sequenced on an ABI 377 automated sequencer (Applied Biosystems) using Big Dye terminators (Applied Biosystems). The status of each amplified CpG was examined for the presence of a cytosine or a thymine in order to determine the methylation status, with a cytosine indicating complete methylation, a thymine indicating

no methylation, and the presence of both nucleotides indicating partial methylation.

3.2.3 Statistical Analysis

Methylation of each tumor and corresponding normal tissue sample was quantified as the number of CpG sites observed to be methylated. The number of CpG sites was the same for each sample. The number of methylated CpG sites was then compared between a tumor sample and the corresponding normal tissue sample using the paired t-test.[18]

3.3 Results

3.3.1 Feline 14-3-3 σ Sequence

575 base pairs of the first exon of feline σ were sequenced. Figure 3.3 shows this sequence as compared to the homologous human σ sequence (Genbank accession AF029081). The entire human σ first exon is 744 bp, as represented by nucleotides 8638 - 9381. Overall, human and feline σ are highly conserved, with 94% nucleotide identity and 92% amino acid identity.

Feline σ contains 37 CpG sites in the sequenced region as compared to 34 in human σ. 27 CpGs are conserved between the cat and the human (73% of the total cat CpGs). There are 10 new CpG sites in the cat, representing 27% of the total cat CpGs. 7 CpG sites appear in the human but not the cat, representing 21% of the total human CpGs. Figure 3.3 illustrates these CpG sites.

Figure 3.3: Human and Feline σ Exon 1. Vertical lines represent conserved nucleotides. Conserved CpG sites are shown in bold and unconserved sites are underlined.

```
Human:8674 GCAGAGCAGGCCGAACGCTATGAGGACATGGCAGCCTTCATGAAAGGCGCCGTGGAGAAG
           |||||||||||||||||||| ||||||||||||||||||||||||  |||||||||| |||
Cat:     1 GCAGAGCAGGCCGAACGCTACGAGGACATGGCAGCCTTCATGAAGAGCGCCGTGGAAAAG

Human:8734 GGCGAGGAGCTCTCCTGCGAAGAGCGAAACCTGCTCTCAGTAGCCTATAAGAACGTGGTG
           || |||||||| ||||||||||||||||||||||||||||| ||||| ||||| ||||||
Cat:    61 GGTGAGGAGCTATCCTGCGAAGAGCGAAACCTGCTCTCAGTGGCCTACAAGAATGTGGTG

Human:8794 GGCGGCCAGAGGGCTGCCTGGAGGGTGCTGTCCAGTATTGAGCAGAAAAGCAACGAGGAG
           |||||||||||||||||||||||| |||||||||| ||||||||| ||||||||||
Cat:   121 GGCGGCCAGAGGGCTGCCTGGAGGGTCCTGTCCAGTATCGAGCAGAAAGGCAACGAGGAG

Human:8854 GGCTCGGAGGAGAAGGGGCCCGAGGTGCGTGAGTACCGGGAGAAGGTGGAGACTGAGCTC
            ||||||| |||||||| || |||||||| ||||||||||||||||||||||||||||
Cat:   181 AGCTCGGAAGAGAAGGGCCCGGAGGTGCGAGAGTACCGGGAGAAGGTGGAGACTGAGCTC

Human:8914 CAGGGCGTGTGCGACACCGTGCTGGGCCTGCTGGACAGCCACCTCATCAAGGAGGCCGGG
           | |||||||| ||||| |||||||||||||||| ||||||||||||||||||||
Cat:   241 CGGGGCGTGTGTGACACGGTGCTGGGCCTGCTGGACACCCACCTCATCAAGGAGGCCGGT

Human:8974 GACGCCGAGAGCCGGGTCTTCTACCTGAAGATGAAGGGTGACTACTACCGCTACCTGGCC
           |||||||||||| |||||||||||||||| |||||||| ||||||||||||||||||
Cat:   301 GACGCCGAGAGTCGGGTCTTCTACCTGAAAATGAAGGGCGACTACTACCGCTACCTGGCT

Human:9034 GAGGTGGCCACCGGTGACGACAAGAAGCGCATCATTGACTCAGCCCGGTCAGCCTACCAG
           |||||||||||| |||||||||||||||||||||||||||| |||||||| |||||||||
Cat:   361 GAGGTGGCCACTGGTGACGACAAGAAGCGCATCATTGACTCGGCCCGGTCCGCCTACCAG

Human:9094 GAGGCCATGGACATCAGCAAGAAGGAGATGCCGCCCACCAACCCCATCCGCCTGGGCCTG
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Cat:   421 GAGGCCATGGACATCAGCAAGAAGGAGATGCCGCCCACCAACCCCATCCGCCTGGGCCTG

Human:9154 GCCCTGAACTTTTCCGTCTTCCACTACGAGATCGCCAACAGCCCCGAGGAGGCCATCTCT
           || |||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Cat:   481 GCGCTGAACTTTTCAGTCTTCCACTACGAGATCGCCAACAGCCCCGAGGAGGCCATCTCG

Human:9214 CTGGCCAAGACCACTTTCGACGAGGCCATGGCTGA
           |||||||||||||| ||||||||||||||||||
Cat:   541 CTGGCCAAGACCACCTTCGACGAGGCCATGGCTGA
```

3.3.2 Feline Mammary Carcinoma Methylation Status

Paired normal and tumor DNA from 5 cases was examined for methylation status of the σ CpG island. Eight unpaired samples (three normal and five tumor DNA) were also examined. Details of each case examined can be found in table 3.1. For each sample, each CpG site was determined to be methylated, partially methylated, or unmethylated. For each statistical calculation, partially methylated sites were counted as 0.5 completely methylated sites counted as 1. Results are summarized in Tables 3.2 (paired samples) and 3.3 (unpaired samples).

For paired samples, the number of methylated CpGs in the normal sample was subtracted from the number of methylated CpGs in the tumor sample in order to calculate the difference between the two. The differences were then analyzed with a *t*-test using SAS software. The two groups were found not to be significantly different (P=0.1671). All samples were then divided into normal DNA and tumor DNA groups and the mean number of methylated CpGs for each group was compared with a *t*-test using SAS software. Again, the two groups were not found to be significantly different (P=0.3565). In conclusion, there is no significant difference in methylation of σ between normal feline mammary tissue and feline mammary carcinomas.

3.4 Discussion

In this study it was found that *14-3-3 σ* is not hypermethylated in feline mammary carcinomas. This is in contrast to human breast tumors, which have been found to have σ hypermethylation in >90% of examined cases.[9] In human breast tumors with hypermethylation, all or almost all CpG sites were found to be completely methylated,

Table 3.1: Cases Examined for σ Hypermethylation. F = female, F/S = female/spayed, NR = not reported.

Case #	Breed	Age (years)	Sex	Tumor Type	Malignancy	Prognosis	Comments
Paired Samples							
10	Mixed	12	F	NR	High	Guarded	High mitotic index, cellular atypia
13	NR	NR	NR	Acinar	High	Poor	Recurring tumor, high mitotic index
121	Mixed	15	F/S	Acinar/Papillary	Low	Guarded	
143	Mixed	16	F/S	Acinar/Solid	High	Poor	Recurring tumor, locally invasive, vascular invasion
158	Mixed	11	F/S	Papillary	Moderate	Guarded	
Unpaired Samples: Normal DNA Examined							
12	Mixed	10	F/S	Acinar	High	Guarded	Locally invasive
16	Persian	12	F/S	NR	NR	Guarded	High mitotic index, cellular atypia
151	Mixed	10	F/S	Papillary	High	Guarded	Locally invasive
Unpaired Samples: Tumor DNA Examined							
130	Mixed	12	F/S	Acinar/Papillary	NR	Guarded	Multinodular, multicystic
133	Mixed	8	F/S	Papillary	High	Guarded	Locally invasive
139	Siamese	7	F/S	Acinar/ Scirrhous	High	Poor	Vascular invasion
149	Mixed	10	F	Solid	High	Poor	Vascular invasion
166	Mixed	8	F/S	Papillary/Solid	High	Poor	Possibly recurring tumor, vascular invasion

Table 3.2: Number of Methylated CpGs in Paired Feline Mammary Carcinoma Samples. P = partially methylated, C = completely methylated.

Case #	Normal Sample	Tumor Sample	Difference
10	0	4P, 1C	4P, 1C
13	4P	4P, 2C	2C
121	0	0	0
143	0	3P	3P
158	3P	2P	-1P

Table 3.3: Number of Methylated CpGs in Unpaired Feline Mammary Carcinoma Samples. P = partially methylated, C = completely methylated

Case #	# Sites Methylated
Normal Samples	
12	0
16	6P, 1C
151	0
Tumor Samples	
130	0
133	IP
139	IP
149	3P, 2C
166	4P

while normal breast tissue exhibited at most one methylated site.[9] In the feline cases examined in this study, a maximum of 2 CpG sites were found to be completely methylated and a maximum of 6 sites were found to be partially methylated out of the 14 total CpG sites analyzed. Unlike human cases, σ methylation in the cat did not correlate with tumor samples: some tumor samples exhibited no methylated CpGs, and the sample with the most methylated sites (six partial and one complete) was from normal tissue.

The process of bisulfite modification and amplification of DNA is technically challenging and therefore the number of samples successfully analyzed for σ methylation

in this study is small. However, the sample population is representative of the larger population of feline mammary tumor biopsies submitted to AHDL, covering a variety of tumor types and characteristics. Several of the samples were highly malignant, increasing the likelihood of identifying σ hypermethylation even if it were a late-occurring event in feline mammary tumorigenesis.

Several questions concerning feline σ and feline methylation are unanswered in this study. It is unknown whether σ is normally expressed in the cat, and the finding that σ is expressed in rat but not mouse mammary tissue indicates there is variation between species.[2] If σ expression does occur in the cat, this study does not completely rule out σ involvement in feline mammary tumorigenesis and further studies need to be done to characterize the role of this gene in the cat. This is the first reported study of hypermethylation and feline carcinogenesis, and more work needs to be done in the cat on this epigenetic phenomenon that plays a role in several human cancers.

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CHAPTER 4

P53

4.1 Background

P53 is an extensively studied tumor suppressor gene. In the human, this approximately 20 kb gene located on chromosome 17pl3 consists of 11 exons and encodes a 393 amino acid nuclear phosphoprotein.[1] The 53 kDa protein was first reported in 1979 as a component of cells transformed by simian virus 40 (SV40).[2] In 1990, germline *P53* mutations were discovered to be the cause of Li-Fraumeni syndrome, an autosomal dominantly inherited disorder in which members of affected families develop one or more types of cancer at an early age of onset.[1] By the 1990s, tumor suppressor genes in general and *P53* in particular were enthusiastically studied by many in order to better understand the development and treatment of cancer, thus earning P53 the title "Molecule of the Year" from Science in 1993.[3] In the year 2003 alone there were 3399 Medline citations found with the keyword search "*P53*".

P53 normally functions as part of the Gl/S checkpoint in the cell cycle, causing cell cycle arrest and cellular apoptosis in the presence of DNA damage. DNA damage induces the expression of P53, which then acts as a transcription factor in the nucleus and affects expression of several other genes including p21, MDM2, and Bax,[4] which in turn regulate the cell cycle and apoptosis. P53 also induces genes involved in the G2/M checkpoint such as *14-3-3* σ. The normal cell only contains a small amount of P53 as the protein has a short half life and is targeted for degradation by the protein MDM2[4]. The accumulation of P53 in a cell, as detected by immunocytochemical staining, is indicative of a missense mutation which stabilizes the protein and reduces its ability to induce

MDM2.[5]

Alterations in *P53* have been found in 50-55% of human cancers.[6] These alterations have been found in a variety of cancers, including 75-80% of colorectal tumors[2] and 30-40% of breast tumors.[7] *P53* may be altered through deletions, insertions, base pair substitutions, chromosomal loss, or other mechanisms affecting tumor suppressor gene expression as discussed in Chapter 1. Many point mutations have been reported, with most being missense mutations resulting in an altered protein (rather than nonsense mutations resulting in a truncated protein). These mutations are clustered between amino acids 130-290, with most occurring within four domains that are highly conserved between many species.[2] Amino acid residues 175, 248, and 273 frequently contain point mutations and have been dubbed mutational "hot spots".[2]

Changes in *P53* have also been extensively studied in spontaneous breast cancer cases in order to determine if the presence of *P53* mutation is useful as a prognostic factor or if it can be used to predict a response to various therapies. Several studies have found that human patients with P53 overexpression have a poorer prognosis.[8] There have been variable results in studies looking at *P53* status and therapy efficacy. Some studies have found that *P53* alteration is predictive for resistance against tamoxifen, doxorubicin, and radiotherapy, while other studies have not found predictive value for these treatments.[9] More studies specifically assessing P53 mutations need to be done in order to obtain a true picture on the usefulness of *P53* status as a factor in therapy selection.

The involvement of *P53* in feline cancers has just begun to be studied. Feline *P53* mRNA has been sequenced (Genbank accession D26608) and shows 86% nucleotide conservation with human *P53* mRNA (Genbank accession NM000546). Feline *P53*

introns 5, 6, and 7 have also been sequenced (Mayr et al.[10], Genbank accessions U81292, and U81298, respectively. See figure 4.1 for sequences.). Using feline x rodent somatic cell hybrids, feline *P53* has been mapped to feline chromosome E1.[11]

Figure 4.1: Feline P53 Genomic Sequence: Exon 5 - Exon 8. Uppercase = exons, lowercase = introns. SNP locations are underlined.

```
   1 TACTCCCCTC CCCTCAACAA GCTGTTTTGC CAGCTGGCGA AGACCTGCCC
  51 CGTGCAGCTG TGGGTCCGAT CGCCGCCCCC ACCGGGAACC TGTGTCCGCG
 101 CCATGGCCAT TTACAAGAAG TCAGAGTTCA TGACAGAGGT CGTGAGGCGC
 151 TGTCCCCACC ACGAGCGCTG CCCTGACAGT AGCGATGggt gagccgtcgg
 201 gggctacaga tggggcaggg cctgctgcta gggtcccccg gcccctgatt
 251 cctccccgat tgctctcagG TCTGGCGCCT CCCCAGCATC TCATCCGAGT
 301 GGAAGGAAAC TTGCATGCCA AGTACCTGGA CGACAGAAAC ACTTTCCGAC
 351 ATAGCGTCGT GGTGCCCTAC GAGCCGCCCG AGgtctgctt tggcatctgg
 401 ggtctctggg aggaggtggg ggaggggttt gtcagcggcc gtccaggtgg
 451 gagatggggg gggctttctc cttcttatgc aacctcccca cggccgcgtg
 501 cggtgtgcac agccagccgg gtggtcccca gtgcacggtt gaggaaacca
 551 gcctacacac tgcaggcctg cccggcgctg ggtggcctca ctcggccgga
 601 tcttctctcc cagGTCGGCT CTGACTGTAC CACCATCCAC TACAATTTCA
 651 TGTGTAACAG TTCCTGCATG GGGGGCATGA ACCGGAGGCC CATCATCACC
 701 ATCATCACCC TGGAAGACTC CAAgtaggga cccgcacgcc accctgcccc
 751 aggccactct ctcccgtgct accgcccatc ccgcctgtgg aatccccgcc
 801 tgtggaatct cctctgctgt cccccaccct ccgcctccaa gttttctttt
 851 ctctggcttt gggaccttct cttacccggc ttctcgatac tccttaggct
 901 tttaggctcc acataggatg aaggaggtgg ggagtaaggg gggccccatc
 951 tccctcactg cctccagctt ctgtcttctt acgtgggtag TGGGAAGCTG
1001 CTGGGACGGA ACAGCTTCGA GGTACGAGTT TGTGCCTGTC CTGGGAGAGA
1051 CCGGCGCACC GAGGAGGAAA ATTTCCGCAA GAAGGGGGAG CCTTGCCCTG
1101 AGCCGCCC
```

Several different types of feline neoplasms have been surveyed to determine if P53 plays a role in feline cancer. Seventy-seven feline tumors of seven different types were examined with immunocytochemical analysis and 20 were positive for P53 staining, including 3 of 9 mammary carcinomas.[5] P53 involvement in vaccine-associated feline sarcomas has been of interest, with one study finding 7 of 18 informative cases (39%) showing loss of heterozygosity[12] and another study reporting 8 of 21 cases (38%)

showing dark P53 immunostaining.[13] One group in particular at the Veterinary University of Vienna, Austria has examined many different feline tumors for P53 mutations through PCR and sequencing. Of the 29 mammary carcinomas this group has reported studying, 3 had mutations.[10,14-17] One had a missense mutation resulting in an arginine to tryptophan amino acid transition at codon 282,[14] one had a missense mutation resulting in an arginine to cysteine amino acid transition at codon 158,[17] and one had a 9 bp deletion affecting codons 251-256.[16]

Loss of heterozygosity (LOH) is a hallmark of tumor suppressor gene involvement in tumorigenesis. A cell needs at least one functioning copy of a tumor suppressor gene to control growth. If that one functioning copy of the gene is lost, then the cell may undergo uncontrolled cell growth and tumorigenesis. In LOH, a heterozygous marker is identified in or near the gene of interest. Marker alleles are compared between normal tissue and tumor tissue. If a tumor tissue marker is homozygous as compared to the heterozygous normal tissue, than LOH has occurred. If one marker allele has disappeared, it is an indication that the functioning tumor suppressor gene allele has been lost, leaving behind a gene copy that is not functioning due to changes such as mutation or hypermethylation.

In order to assess loss of heterozygosity, one must identify a heterozygous marker in or near the tumor suppressor gene of interest. Single nucleotide polymorphisms, or SNPs, have become a very useful marker for this purpose. A SNP is a locus in genomic DNA in which different alleles exist for a single base pair in normal individuals of a population. SNPs are plentiful, with more than 1,400,000 SNPs reported in the NCBI database for the human genome and more being published all the time. Often SNPs are

located in introns or other non-coding regions, thus creating silent changes that can be genotyped through methods such as sequencing or assessing restriction site changes.

A study concentrating on the role of *P53* alterations in feline mammary carcinoma has not been published. As feline mammary carcinomas are similar to human breast tumors, I propose that the two cancers have a similar rate of *P53* loss of heterozygosity.

4.2 Materials and Methods

4.2.1 Development of SNP Genotyping Tests

Genbank and the literature were searched for polymorphisms within feline *P53*, listed in table 4.1. The reported minor allele frequencies were calculated from small European sample populations. Polymorphism base pair positions are given according to the nucleotide numbering in figure 4.1.

Table 4.1: Reported Polymorphisms in Feline *P53*. NR = Not Reported.

Polymorphism	Location	Position	Minor Allele Frequency	Reference
C / T SNP	Exon 5	114	T = NR	Mayr, 1995[10]
C / T SNP	Intron 6	495	T = 0.2	Mayr, 1998[16]
C / T SNP	Intron 7	737	T = 0.5	Mayr, 1998[16]
T / C SNP	Intron 7	969	C = NR	Kanjilal, 1999[12]
T insertion	Intron 7	970	T insertion = NR	Genbank AF175762
C / T / G SNP	Intron 7	982	T = 0.5, G = NR	Mayr, 1998[16]; Kanjilal, 1999[12]

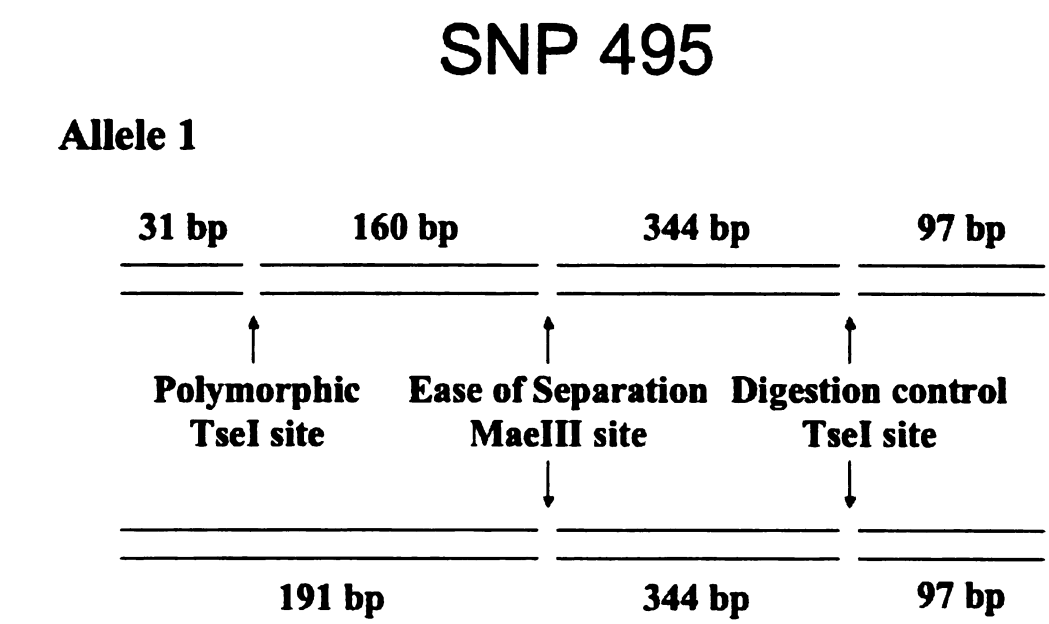
Restriction enzymes recognize specific short sequences of DNA and cleave the

DNA at this restriction site. The polymorphisms were examined to determine if they were part of a restriction site. Polymorphisms 495, 737, and 970 had one allele that created a restriction site while the other allele did not. The details of the genotyping test for each polymorphism are given in section 4.3.1, but the general design plan is as follows. Primers were designed to amplify a region of DNA by PCR containing the polymorphic restriction site. In order to provide a control for the restriction enzyme digestion, the amplified fragment also contained a restriction site that does not contain a polymorphism and is thus always cleaved. The amplified DNA was then incubated with the proper restriction enzyme overnight and the size of the resulting fragments were examined by agarose gel electrophoresis. Fragment sizes differ depending on whether or not the restriction enzyme was able to cleave the polymorphic site, thus providing a rapid, reliable, and inexpensive method of genotyping polymorphisms. Figure 4.2 illustrates the design of the restriction digest genotyping tests.

4.2.2 Allele Frequencies

The polymorphisms selected for study either did not have allele frequencies reported or had allele frequencies reported for a small number of samples from a European cat population. In order to determine allele frequencies for a North American cat population, buccal cell sampling using cytology brushes was performed on cats belonging to Michigan State University College of Veterinary Medicine staff and students. Four buccal samples were performed by the owner of each cat. DNA was isolated from one swab per cat and the other three stored for archival purposes. In order to isolate genomic DNA from each swab, the swab was placed in a 1.5 ml eppendorf tube

Figure 4.2: Schematic of Restriction Digest Genotyping Tests.



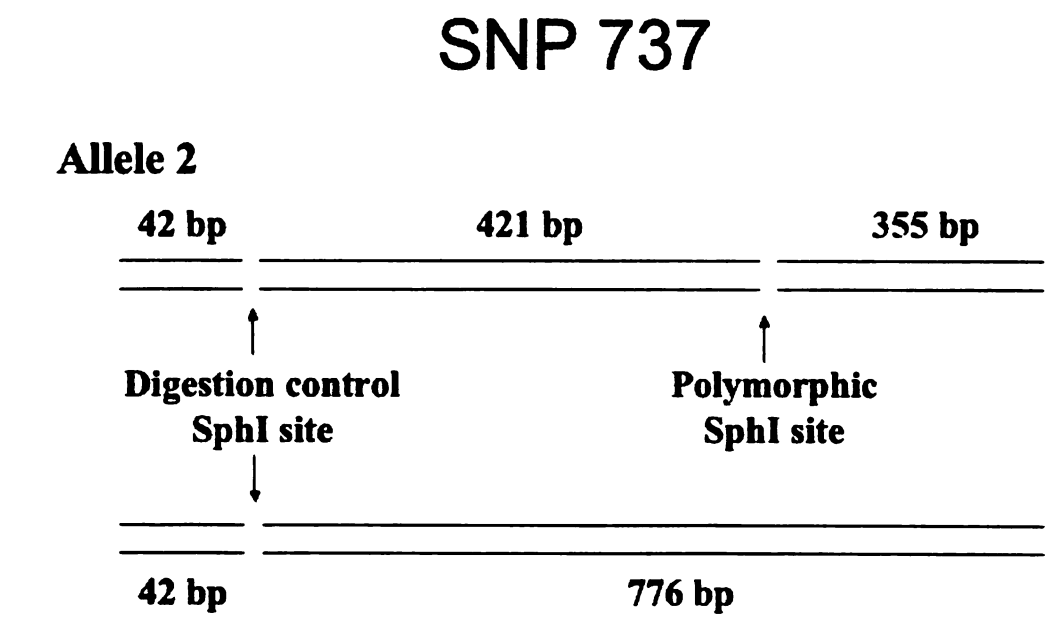
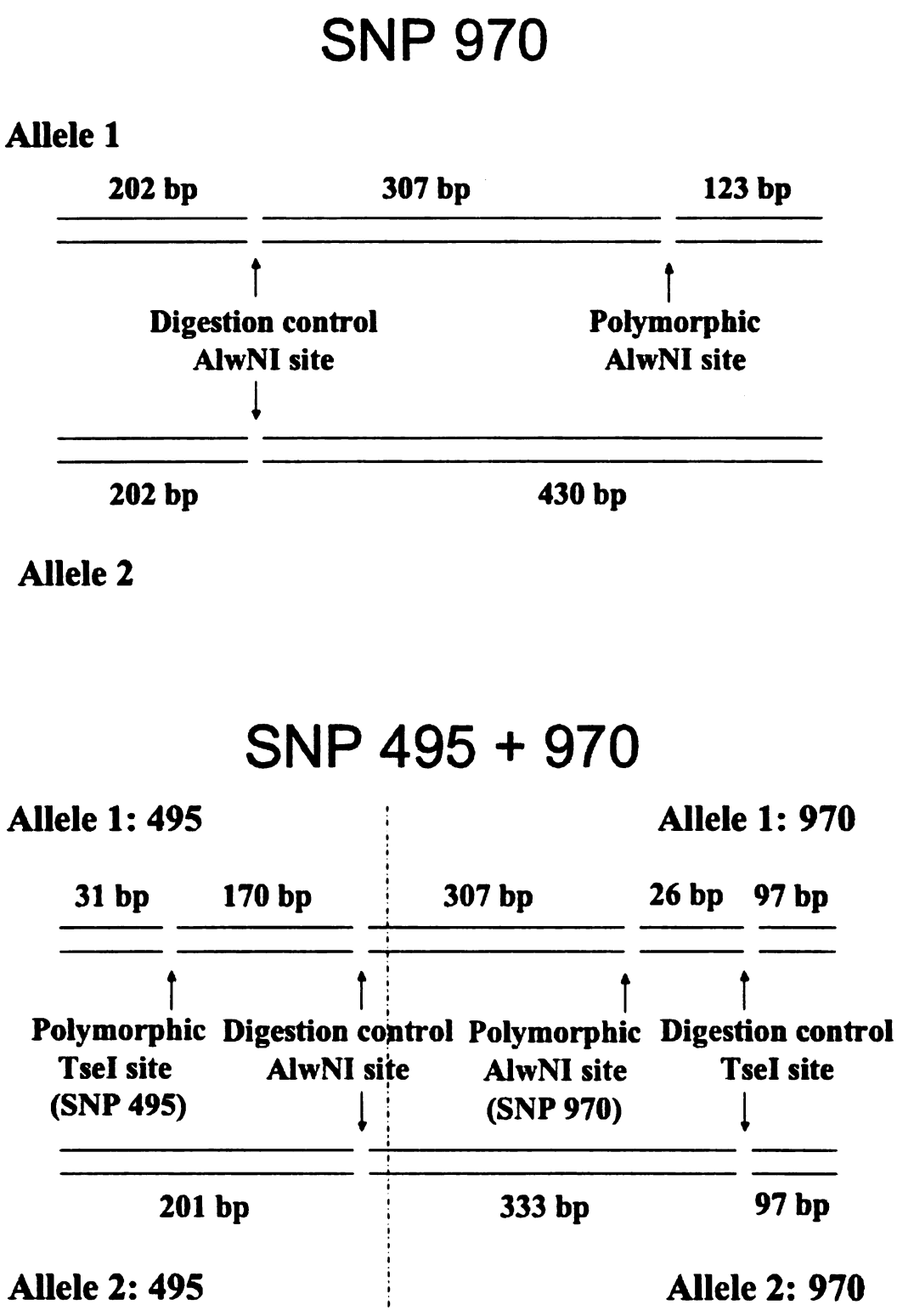


Figure 4.2 continued: Schematic of Restriction Digest Genotyping Tests.



and immersed in 600 µl of 50 mM NaOH. The tube was vortexed and heated to 95 °C for 5 minutes and vortexed again with brush still in the tube. 60 µl of 1 M Tris (pH 8.0) was added to neutralize the solution. The tube was vortexed again and stored at 4 °C with the brush still in the tube. 4-10 µl of this solution was used as template in a 25 µl PCR reaction.

These samples were genotyped for the three selected *P53* polymorphisms described above. Each sample was amplified by PCR and digested with a restriction enzyme as detailed in tables 4.2 and 4.3.

Table 4.2: SNP Genotyping Tests: PCR Conditions

SNP	Primers (Forward/Reverse)	Template (µl)	Primer (µM each)	$MgCl^2$ (mM)	Taq (U)	Anneal. Temp. (°C)	Cycles	PCR Product Size (bp)
495	5' GGCTTTCTCCTTCTTATGCAACCT 3' 5' AAGGCTCCCCCTTCTTGCGG 3'	4	0.4	1.5	2.5	66	40	632
737	5' CGCCTCCCCAGCATCTCATC 3' 5' AAGGCTCCCCCTTCTTGCGG 3'	10	0.6	2	2.5	70	35	818
970	5' GGCTTTCTCCTTCTTATGCAACCT 3' 5' AAGGCTCCCCCTTCTTGCGG 3'	4	0.4	1.5	2.5	66	40	632
495 + 970	5'GGCTTTCTCCTTCTTATGCAACCT 3' 5' AAGGCTCCCCCTTCTTGCGG 3'	41	0.4	1.5	2.5	66	40	632

4.2.3 Genotyping of Feline Mammary Carcinoma Samples

Normal mammary tissue DNA, isolated from feline mammary carcinoma samples as described in Chapter 2, was genotyped for one or more selected polymorphisms as described in sections 4.2.1 and 4.3.1. Samples were considered informative if the normal tissue was heterozygous at one or more polymorphic loci. Tumor DNA from informative samples was genotyped and the alleles present compared to those of normal tissue DNA. Samples were then categorized as showing no LOH, partial LOH, or complete LOH.

Samples with no LOH were heterozygous in both the normal and tumor DNA. Samples with LOH were heterozygous for a given polymorphism in the normal DNA but

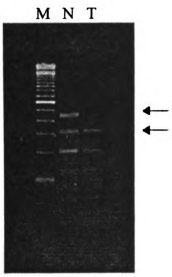
Table 4.3: SNP Genotyping Tests: Restriction Digests

SNP	Restriction Enzyme (μl)	Additional 50 mM $MgCl_2$ (μl)	Incubation Temp	Agarose Gel %	Band Sizes (bp)		
					Allele 1	Allele 2	Heterozygote
495	TseI = 0.5, MaeIII = 1.0	2.2	55 °C	3 %	31, 97, 160, 344	97, 191, 344	31, 97, 160, 191, 344
737	SphI = 1.0	2.0	37 °C	2 %	42, 776	42, 355, 421	42, 421, 355, 776
970	AlwNI = 0.5	2.0	37 °C	1.5 %	123, 202, 307	202, 430	123, 202, 307, 430
495 + 970	AlwNI = 0.5, TseI = 0.5	2.0	37 °C	3 %	For 495: 31, 170	97, 201	31, 97, 170, 201
					For 970: 26, 97, 307	97, 333	26, 97, 307, 333

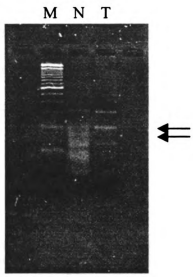
homozygous in the tumor DNA. Samples with partial LOH were heterozygous in both the normal and tumor DNA, although the tumor DNA showed allelic imbalance. When visualizing bands formed by differently sized DNA fragments on an agarose gel, bands representing both alleles in a heterozygous sample normally appear as approximately the same brightness, indicating approximately the same amount of DNA in each band. However, the bands are of different brightness in a sample with allelic imbalance, indicating different amounts of DNA in each band. Therefore, a sample with allelic imbalance contained a mixed population of cells: some cells have undergone LOH while others have not. Figure 4.3 illustrates allelic imbalance.

Figure 4.3: Complete LOH versus Allelic Imbalance. M = DNA marker, N = normal tissue, T = tumor tissue

A) Complete LOH



B) Allelic Imbalance



4.3 Results

4.3.1 SNP Genotyping Tests

A total of four restriction digest genotyping tests were designed. Three tests genotype a single polymorphism (495, 737, and 970) while one test genotypes two polymorphisms at once (495 and 970). The general plan for these tests was described previously in section 4.2.1, while specific details for each test can be found in table 4.2. A typical PCR reaction contained 4-10 µl DNA template, 1.5-2 mM $MgCl_2$, 0.12 mM dNTPs, 0.4-0.6 µM each forward and reverse primer, 1x buffer, and 2.5 U Taq polymerase. Each reaction was denatured at 94°C for 4 minutes; cycled through 94°C for 1 minutes, 66°C or 70°C for 2 minutes, and 72°C for 3 minutes for 35-40 cycles; and incubated at 72°C for 8 minutes in a thermocycler. 10 µl of the PCR product was then

combined with additional $MgCl_2$ and restriction enzyme, incubated overnight, and examined by agarose electrophoresis (see Table 4.3 for details for genotyping each SNP).

4.3.2 Allele Frequencies

Allele frequencies for each of the three SNPs genotyped are reported in table 4.4. Allele frequencies for both the CVM reference cat population and the feline mammary carcinoma samples are reported. When the allele frequencies of the two groups were compared with chi square analysis there were no significant differences between them.

Table 4.4: SNP Allele Frequencies

SNP	Group	# Samples	Major Allele	Minor Allele	Hetero. Obs.	Hetero. Calc.
495	Ref	62	0.62	0.38	0.50	0.47
	Test	30	0.62	0.38	0.43	0.47
737	Ref	20	0.62	0.38	0.25	0.47
	Test	29	0.71	0.29	0.17	0.41
970	Ref	84	0.91	0.09	0.16	0.17
	Test	29	0.86	0.14	0.28	0.24

4.3.3 Loss of Heterozygosity of P53

28 normal samples were genotyped at one or more SNPs in order to obtain 20 informative cases, for an informative rate of 71%. A summary of results is found in table 4.5. A total of six samples showed complete or partial LOH, a rate of 30%. A summary of cases studied is shown in table 4.6.

Table 4.5: P53 LOH Results

Category	# Samples	Percentage
No LOH	14	70
LOH	3	15
Allelic Imbalance	2	10
LOH at 1 of 2 Informative Loci	1	5
Totals	20	100

4.4 Discussion

The SNP genotyping tests presented are a rapid, inexpensive, and reliable way to examine feline *P53* LOH. They are used here to study feline mammary carcinomas but will be useful in the investigation of *P53* LOH in other feline cancers. Genotyping tests for three different SNPs increases the number of informative cases available for study. The combined 495/970 SNP test is useful in genotyping two SNPs at once, but if the results from this test are unclear, then the presented alternative tests for each single SNP can be used.

The LOH rate of 30% is similar to the 30-40% *P53* LOH rate in human breast tumors.[7] This similarity is not unexpected as *P53* is highly conserved in structure and function between species, with P53 playing a critical role in the control of the cell cycle. The results of this initial study encourage further investigation into the role of P53 in feline mammary carcinoma. If more similarities between the role of *P53* in feline and human mammary cancers are found, treatments developed for *P53* deficient human tumors may be applied to feline cancer patients.

This study did not contain a large enough sample size to demonstrate a statistically significant correlation between *P53* LOH and tumor invasiveness, metastasis,

Table 4.6: Cases Studied for P53 LOH. N.R. = Not reported.

Status	Breed	Age	Sex	Tumor Type	Malig.	Prognosis	Comments
LOH	Persian	12y	F/S	N.R.	High	Guarded	
LOH	Mixed	N.R.	N.R.	Solid/acinar	High	Poor	Vascular invasion
LOH	Mixed	8y	F/S	Solid/papillary	High	Poor	Vascular invasion
Partial LOH	Mixed	12y	F/S	Acinar	High	Guarded	Locally invasive
Allelic Imbal.	Mixed	9y	F/S	Acinar	High	Guarded	Locally invasive
Allelic Imbal.	Mixed	11y	F/S	Papillary	High	Poor	L.N. metz., recurring tumor
No LOH	Mixed	11y	F/S	Papillary	Mod.	Guarded	
No LOH	Mixed	10y	F/S	Solid	Low	Guarded	
No LOH	Mixed	18y	F/S	Papillary	Low	Guarded	
No LOH	Mixed	16y	F/S	Solid/acinar	High	Poor	Locally invasive, vascular invasion, recurring tumor
No LOH	Siamese	7y	F/S	Acinar/Scirrhous	High	Poor	Vascular invasion
No LOH	Maine Coon	9y	F/S	Acinar	N.R.	Guarded	
No LOH	Mixed	8y	F/S	Papillary	High	Guarded	Locally invasive
No LOH	Mixed	13y	F	Acinar	High	Poor	Vascular invasion
No LOH	Mixed	15y	F/S	Acinar/papillary	Low	Guarded	
No LOH	Mixed	18y	F	Acinar/mixed	High	Poor	Vascular invasion, metz on radiographs
No LOH	Mixed	7y	F	Papillary	Low	Guarded	
No LOH	Mixed	13y	N.R.	Papillary	N.R.	Guarded	
No LOH	Mixed	15y	F	Acinar	N.R.	Guarded	
No LOH	Mixed	10y	F	Papillary	Mod.	Guarded	

or prognosis. However, all cases found to exhibit *P53* LOH were described as highly malignant while all cases of low or moderate malignancy did not exhibit *P53* LOH. Therefore, with further study, *P53* LOH may prove to be a useful prognostic indicator in feline mammary carcinomas. As there is no currently accepted histological grading system for these tumors that correlates with prognosis, especially for moderately differentiated carcinomas, a molecular indicator of prognosis would be a useful tool for clinicians.

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CHAPTER 5

CONCLUSION

5.1 Potential Gene Targets in Future Studies of Feline Mammary Adenocarcinoma

Understanding the molecular genetic changes that occur during tumorigenesis has become increasingly important in human oncology; molecular markers are now being used as prognostic indicators and treatment targets. However, molecular changes in veterinary cancer patients have not received the same attention. To begin studying molecular markers of feline mammary carcinomas, two genes that are associated with human breast tumors were selected. *P53* was selected as it has been extensively studied and is thought to play a role in tumorigenesis in many types of human cancers, including breast cancer. On the other hand, σ has only recently been implicated in human breast cancer. However, changes in this gene were found in a high percentage of tumors examined and were being investigated as a target of therapy, making it an interesting gene to examine in another species such as the cat.

P53 and σ represent only a fraction of the genes that have been studied in human breast cancer, leaving many more to investigate in feline mammary carcinomas. One such gene is *HER2*, also called *ErbB2* or *neu*, a tyrosine kinase receptor that has been found to be overexpressed in 20-40% of human breast cancers.[1] The most common mechanism of overexpression of *HER2* is gene amplification, in which several copies of a gene or chromosomal region are present. In humans, *HER2* amplification is associated with aggresive tumor behavior, shorter suvival time, and overall poor prognosis.[2] *HER2* overexpression is also predictive of response to some types of therapy. *HER2*+ tumors are resistant to hormonal therapies such as tamoxifen but have increased sensitivity to the

chemotherapeutic agent anthracycline.[3] Recently, *HER2* itself has become a therapeutic target: trastuzumab, a humanized anti-HER2 monoclonal antibody, has been approved by the FDA for treatment of women with HER2 + breast tumors.[3]

The first step in investigating *HER2* in the cat would be to determine the feline nucleotide sequence for this gene. The same process used to determine the feline σ sequence could be used to determine the feline *HER2* sequence. Currently, the *HER2* genomic sequence has been determined for the human (Gen-bank accession NM_004448) and the mouse (Genbank accession NT_031413.2) while the mRNA sequence has been determined for the rat (Genbank accession X03362). This existing sequence information could be used to design oligonucleotide primers that would bind to highly conserved regions of *HER2*, which could then be used to amplify and sequence feline *HER2*. Once feline *HER2* is sequenced, current methods used to evaluate human *HER2* amplfication/overexpression could be assessed for their usefulness in the cat. One method commonly used to assess *HER2* status in human breast cancer patients is immunohistochemistry (IHC), in which mammary tissue slides are stained with an antibody which binds to HER2 protein. In order to visualize the antibody binding sites, the antibody itself may carry a marker such as fluorescein or horseradish peroxidase, or a secondary antibody carrying a marker which then binds to the primary antibody may be used. The slide is then visually examined for presence of the marker. There are currently more than 30 anti-human HER2 antibodies as well as a commercial kit (HercepTest, DAKO) which are available for IHC.[4] If feline and human HER2 are sufficiently similar, then these antibodies may be used to perform IHC on feline mammary tissue and tumor samples and assess HER2 overexpression. However, if anti-human HER2 antibodies do

not bind to feline HER2, the prohibitive time and expense involved in generating anti-feline HER2 antibodies makes IHC a less practical techinique for examining feline HER2 overexpression. Fluorescence in situ hybridization (FISH) is another technique that is currently being used to detect human HER2 amplification. In FISH, a slide-mounted tissue section is stained with a fluorescently labeled oligonucleotide probe which binds to *HER2* in the cells' chromosomes. The slide is then visually examined for the intensity of the fluroescent signal in order to determine *HER2* copy number.[5] Although feline-specific oligonucleotide probles are more easily manufactured than feline-specific antibodies, the specialized equipment necessary to perform FISH may limit the use of this technique in some laboratories. Although less sensitive than FISH, techniques such as differential PCR or Southern blots may also be used to determine if *HER2* is amplified in feline mammary tumor samples. In differential PCR, the target gene and a reference gene are co-amplified by PCR and the product amounts measured by densitometry. The ratio of the target to reference gene product represents the amount of DNA originally in the sample.[6] In Southern blot, DNA is isolated from cells, cleaved into fragments by restriction enzymes, and the fragments are seperated through gel elecrophoresis. The DNA is then transferred to a nitrocellulose membrane and washed with a solution containing a radiolabled oligonucleotide probe which will hybridze to the gene of interest. Radiograph film is then exposed to the nitrocellulose membrane, allowing the fragment size and amount of DNA present to be measured. Most of the above techniques have been shown to be effective with formalin-fixed paraffin embedded samples, allowing examination of the feline mammary carcinoma samples we have already collected.

BRCA1 and *BRCA2* are two other genes that should be investigated in feline mammary cancers. These genes have been associated with familial breast and ovarian cancers, in which a germline mutation in either *BRCA1* or *BRCA2* is passed on from generation to generation, increasing the risk of mammary tumor development. Although somatic mutation is very rare in sporadic human breast cancer cases, loss of heterozygosity is common: 50-70% of sporadic ovarian and breast tumors have LOH of *BRCA*1 and 30-50% have LOH of *BRCA2*.[7] The functions of BRCA1 and BRCA2 proteins are closely related, both being involved in control of homologous recombination and DNA double-strand break repair.[8]

Although familial mammary cancer has not been reported in the cat, the role of *BRCA1* and *2* in sporadic feline mammary cancer should be investigated. LOH of *BRCA1* and *2* can be studied in much the same way as *P53* LOH was studied in this project. The first step would be to obtain the nucleotide sequence of the feline *BRCA1* and *2* genes, which could be done in much the same was as the σ sequence was obtained. A 2845 bp partial coding sequence for feline *BRCA1* has been determined (Genbank accession AF284018) which provides a good starting point for oligonucleotide primer design for PCR. Feline *BRCA2* has not been sequenced, but published *BRCA2* sequences for several other species (including human, mouse, and dog, Genbank accessions NM_000059, NM_009765, and AB043895, respectively) provide information to find conserved sequences on which to base PCR primer design. As there are no published SNPs or other genetic markers for feline *BRCA1* and *2*, the next step would be to identify these genetic markers in the genes of interest. In order to identify SNPs, a pool-and-sequence method can be used. In this method, DNA from several individual cats is combined, the DNA

region of interest is sequenced, and SNPs are identified through the presence of multiple nucleotides at a single locus.[9] Once SNPs are identified, restriction enzyme tests similiar to those used in the *P53* portion of this study can be developed in order to distinguish between SNP alleles. The same feline mammary tissue DNA samples that were used in this study can then be examined for SNP genotype. Once normal mammary tissue samples that are heterozygous at a SNP locus are identified, the corresponding tumor DNA sample can be genotyped in order to determine if it has undergone LOH.

5.2 Methylation in Feline Mammary Adenocarcinoma

In this study, we did not find hypermethylation of 14-3-3 σ in the cat. One possibility is that hypermethylation does not occur in the cat at all, as there have been no other published studies documenting any hypermethylation in this species. However, this lack of reporting is probably due to the scarcity of studies involving genetic and epigenetic phenomena in the cat rather than a lack of feline hypermethylation. As methylation is a highly conserved process and hypermethylation has been documented in other species such as the rat and the mouse, it is probable that hypermethylation does occur at other loci in the cat.

Hypermethylation of several genes other than σ has been linked to human breast cancer. E-cadherin (or E-cad) is a cell adhesion molecule, downregulation of which has been linked to invasion and metastasis in various human carcinomas. E-cad contains a CpG island located in the promoter through intron 1 of this gene. Hypermethylation of this CpG island has been found in greater than 50% of human primary breast tumors examined.[10] Hypermethylation of the *BRCA1* promoter has also been identified. One

study found that 13% of human primary breast tumors had hypermethylation of *BRCA1*.[11] This same study also examined the relationship of *BRCA1* LOH and hypermethylation; 20% of tumors exhibiting LOH were hypermethylated while only 5% of tumors not exhibiting LOH were hypermethylated.

Both *E-cad* and *BRCA1* would be interesting loci to study hypermethylation in feline mammary carcinoma. These genes could be examined in much the same way σ was examined for hypermethylation, using the same DNA samples already isolated from feline mammary tissue and tumors. In order to design PCR primers, conserved regions from the promoter sequences of the human (Genbank accession L34545 (*E-cad*), L78833 (*BRCA1*)), mouse (Genbank accession M81449 (*E-cad*)), rat (Genbank accession AF080590 (*BRCA1*)), and dog (Genbank accession AF330163 (*E-cad*)) can be used. DNA samples can be treated with sodium bisulfite and sequenced as described in chapter 3 in order to determine if the promoters of these genes are hypermethylated in feline mammary carcinoma cases.

5.3 Cats as Animal Models for Human Breast Cancer

Cats have been proposed as a good animal model for human breast cancer as the pathology, behavior, and drug response of mammary tumors is very similar between these species. Molecular genetic changes in breast tumors have been extensively studied in the human but not the cat, making this an unknown factor when evaluating the cat as an appropriate animal model. In this study, we examined two molecular genetic changes that have been documented in human breast tumors but had not previously been studied in the cat. In the case of 14-3-3 σ, cats and humans were very dissimilar- greater than

90% of human primary tumors exhibited hypermethylation while none of the feline tumors exhibited hypermethylation. In the case of *P53*, cats and humans were very similar-mammary tumors of both species had an approximately 30% LOH rate. These disparate results illustrate why careful consideration is necessary when selecting an animal model if the goal of the study is obtain results applicable to human disease. Caution is also needed when applying therapies developed for human disease to veterinary species, as dissimilarities at the molecular level may result in treatment failure. More information is needed concerning molecular genetic events of tumorigenesis in animals in order to develop better animal models for human disease as well as to allow veterinary medicine to take advantage of advances in human disease treatment.

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