Dissertation

Protein Expression of HER-2, CAV-1 and ER alpha in Canine Mammary Tumors and Canine Osteosarcoma

Submitted By

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY Alfred F Flint ENTITLED Protein Expression of HER-2, CAV-1 and ER alpha in Canine Mammary Tumors and Canine Osteosarcoma BE ACCEPTED AS FULFILLING, IN PART, REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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Abstract of Dissertation

Protein Expression of HER-2, CAV-1 and ER alpha in Canine Mammary Tumors and Canine Osteosarcoma

Human Epidermal Growth Factor 2 (HER-2, *neu or erbB-2*) is a protein that influences cell proliferation, morphological differentiation, and cell motility. Additionally, over expression of HER-2 has been shown to promote growth and invasion of cells of mammary neoplasia in vivo. Over expression of HER-2 has been identified in 25-30% of human and canine mammary neoplasms and osterosarcomas though the prognostic significance remains unclear. HER-2 over expression in human breast cancer correlates with a more aggressive tumor type, poor prognosis and resistance to chemotherapeutic agents. However, patients with breast cancer over-expressing HER-2 have benefited from anti-HER-2 therapy. By targeting HER-2, cell proliferation is subsequently inhibited by blocking intracellular signaling with direct targets to the cell cycle machinery.

The studies presented examine the complex protein interactions of HER-2 in mammary neoplasia and osteosarcoma in canine patients. Real-time RT-PCR was used to evaluate HER-2 expression in 7 canine OSA cell lines and 10 canine OSA tissue samples. HER-2 is significantly over expressed in 86% (6/7) of the cell lines and 40% (4/10) of the OSA tissues samples. Given the importance of HER-2 in human breast cancer, the finding of HER-2 over expression in canine OSA may be important in further understanding the pathogenesis and possible therapies of OSA.

Histomorphologic characterization and immunohistochemical analysis of HER-2, caveolin 1 (CAV-1), and estrogen receptor alpha (ERα) was performed on 144 canine

mammary tumors from 44 different breeds. HER-2 was over expressed (score 3) in 23.4% (85/137) of the lesions. Patients with lesions over-expressing HER-2 had a 109d decrease in the median time to reoccurrence and a 276d decrease in median survival time. CAV-1 showed little or no expression in 31% (45/139) of lesions. However when CAV-1 was over expressed (score 2-3) patients had a decrease in the median time to reoccurrence of 236d and a decrease in median survival time of 292d. Finally, ERα expression in the cytoplasm was correlated to lesions that were classified as benign. The immunohistochemical evaluation of HER-2, CAV-1, and ERα support their use in prognostic evaluation.

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Chapter 1

HER-2: A critical player in intracellular signaling in canine and human mammary carcinoma and osteosarcomas

MOLECULAR ORGINIZATION and STRUCTURE of HER-2

Human Epidermal Growth Factor 2 (HER-2, *neu or erbB-2*) is one member of a family of epidermal growth factor receptor (EGFR) tyrosine kinases that includes human epidermal growth factor 1 through 4 (HER-1, HER-2, HER-3 and HER-4).[104] These receptors are responsible for cell proliferation and differentiation in the developing embryo as well as in adult tissue.[49,70] Additionally, aberrant expression of HER-2 is associated with neoplastic lesions in adults.[51,70,96] HER-2 is a 185 kD glycoprotein receptor containing 630 amino acids in four extracellular domains[13,49] which include a tandem repeat of a two domain unit, a single membrane spanning region and a cytoplasmic tyrosine kinase.[11] HER-2, unlike other members of the EGFR family, has a constitutively open ligand binding site. This may explain why there is only one identified high affinity HER-2 ligand, therefore HER-2 serves as a co-receptor with other *erbB* family members.[13] Dimerization in the EGF family of tyrosine kinases is usually mediated via the loop II domain, allowing for the domain III loops to overlap and domain IV to come into position for receptor signaling.[13] However, HER-2 seems unable to create homodimers preferring instead to form heterodimers with other EGF receptors. The lack of HER-2 homodimers is in part due to an Arg to Leu 285 switch in loop II, the only residue not conserved in that domain.[69] This implies that the loop II domain is the most likely candidate for HER-2 heterodimer interaction.[13]

NORMAL HER-2 EXPRESSION

HER-2 is expressed on the basolateral membrane of polarized epithelium and has important functions in organ development which is underscored by elevated HER-2 at the onset of organogenesis.[17,43,76] Moreover, in the rat embryo increased expression of HER-

2 is found in the germinal zones of the developing brain[45] and is critical to the heart-valve mesenchyme formation.[10] In the mouse nervous system, HER-2 influences both the number and migration of neuronal crest cells with HER-2 null mice showing a loss of neuronal crest cells at the forelimb level resulting in sympathetic ganglia aplasia.[8] Furthermore, the absence of HER-2 results in a disorganized innervation of the inner ear in mice.[63] HER-2 is also critical in the development of Schwann cells,[8] which produce critical survival factors for the motor and sensory neurons, and in the terminal differentiation of oligodendrocytes. HER-2 null oligodendrocytes that do manage to differentiate fail to ensheath nearby neuritis.[73] Normal female sexual differentiation of the hypothalamus[77] is dependent on neuronal-astroglial HER-2 expression. HER-2 expression has also been implicated in androgen-independent prostatic cancer growth in men.[99] Moreover, skin, intestine, kidney[44] and lung tissues show increased expression of HER-2 in all stages of development and in adults. Normal human lung fibroblasts express ligands for HER-2, and HER-2 stimulation is required for maintaining epithelial differentiation particularly in type II alveolar cells.[98]

It is difficult to study the developmental effects of the loss of *erbB-2*/HER-2 expression in late gestation because *erbB-2*/HER-2 knockout mice die at midgestation due to severe cardiac defects.[12,49,67] All four erbB receptors are expressed in normal mammary gland development, with *erbB-1* being expressed in all stages of mammary gland development[25] where it has a major role in duct development.[102] *erbB-2/* HER-2 is also expressed in all stages of breast development during normal ductal growth.[41] Transgenic mice expressing a mammary-specific truncated non-functional form of the *erbB-2*/HER-2 gene fail to develop active lobuloalveoli, produce no lobuloaveoli

secretory product[41] and had reduced ductular branching patterns.[4] Additionally, *erbB-2*/HER-2 knockout mice show decreased terminal end bud penetration into the mammary fat pad.[37]

REGULATION of HER-2

Currently, the only proposed HER-2 specific ligand is neu protein specific activating factor (NAF). NAF is similar to EGF in that it activates tyrosine kinase, causing receptor dimerization and internalization.[18] However, there is new evidence that HER-2 is incapable of interacting directly with the high affinity ligand.[13] It is thought that this receptor, locked into an open structure, does not require ligand binding to become active but rather is readily available as a co-receptor for the other EGFR members, hence heterodimerization. Moreover, HER-2 is not autoinhibited and can act in a tumorigenic manner when over-expressed in a cell. These unique properties of HER-2 are underscored by the fact that HER-1 and HER-3 are not known to contribute to tumorigenesis in this manner unless their ligand is present.[13]

Of interest is the fact that the canine HER-2 gene maps to a chromosome region frequently affected by clonal chromosomal aberrations in tumors of the dog.[64] The promoter region of HER-2 contains many interesting features including TATA box, a CAAT box and two Sp-1 transcription factor binding sites.[34] The CAAT and TATA boxes regulate three downstream promoters.[34] In constrast to HER-2, EGFR contains only Sp1 transcription factor binding sites.[35]

The transcription factor AP-2 (OB2-1), binds and activates the *erbB-2*/HER-2 promoter. When the AP-2 binding site is mutated, HER-2 expression is significantly reduced.[31] AP-2 transcription factors play a pivotal role in cell survival, proliferation and

differentiation in the embryo and tumorigenesis.[39] AP-2s role in tumorigenesis has been observed in mammary tumor epithelial cells which overexpress *erbB-2*/HER-2,[7] thus the AP-2 transcription factor may act to promote tumor progression by way of HER-2 over-expression.[38]

Upregulation of phospholipase Cδ-4 (PLCδ-4) is associated with over-expression of HER-2 and EGFR.[52] The regulation of HER-2 by PLC δ-4 is likely due to the resulting diacylglycerol (DAG) from PLC δ-4 hydrolysis of phosphatidylinositol bisphosphate (PIP_2) targeting an important serine/threonine protein kinase C (PKC) which leads to the over-expression of HER-2 and EGFR. The resultant over-expression of HER-2 and EGFR leads to activated extracellular related signaling kinases 1 and 2 (ERK 1/2) which are associated with tumorigenesis (Figure 1).[52] It is well known that human mammary tumor patients with positive HER-2 tissue expression had a significantly shorter survival when compared to those with no HER-2 expression.[78]

DOWNSTREAM SIGNALING of HER-2

HER-2, like other growth factors, increases intracellular Ca^{++} concentrations which accelerate the hydrolysis of polyphosphoinositides.[72] Briefly, this mechanism results from the activation of phospholipase C (PLC) by an EGFR tyrosine kinase heterodimer which causes PLC to cleave PIP_2 to inositol triphosphate (IP_3) and DAG. IP_3 then binds to IP_3 gated Ca^{++} channels on the endoplasmic reticulum which increases intracellular Ca^{++}. The increase in Ca^{++} alters PKC so that it becomes membrane associated and can be activated by DAG. Activated PKC is then able to act on target proteins within the cell.[3] Additionally, activated HER-2 heterodimers interact with several second messenger proteins including phosphatidylinositol 3-kinase (PI_3-

kinase), PLC and GTPase.[74] Activation of HER-2 results in a rapid association with PI_3-kinase and, like other oncogene proteins (i.e. src, abl), uses PI_3-kinase as an effecter molecule.[74] In addition to interactions with second messengers, HER-2, via the phosphokinase B (PKB) pathway, stimulates cyclin D translation and cell proliferation.[46] Specifically HER-2 phosphorylates S6 Kinase (S6k) via protein kinase B. The activated S6k stimulates the S6 ribosomal subunit to increase the translation of ribosomal components. Consequently, the activated S6k acts on a 40S ribosomal subunit that activates cyclin D1 translation.[46]

HER-2 drives epithelial malignancies by forming heterodimers with one of the EGFR receptors and activating the PI_3-kinase/Akt pathway which results in the stimulation of cell growth.[88] Activated PI_3-kinase then activates the serine/threonine kinase of Akt/protein kinase B.[27,94] Activation of Akt inhibits apoptosis and promotes cell survival of mammary epithelial cells by targeting members of the forkhead transcription factor subfamily, specifically FOXO1a, FOXO3a (FKHRL1), and FOXO4.[9,33] When phosphorylated, the forkhead transcription factors become inactive and sequestered in the cytoplasm promoting cell survival and proliferation.[9] When there is an absence of growth factors such as HER-2, FOXO3a and other forkhead factors are able to enter the nucleus and activate death genes which promote apoptosis.[9,66] Additionally, IP3-receptors act as substrate for AKT phosphorylation. When IP3-receptors are phosphorylated the apoptotic effects of Ca^{++}.[42] Neoplastic mammary cells produce high levels of nitric oxide (NO) that downregulate the action of ROCK kinase, an upstream regulatory protein of FOXO3a. Thus when NO is high, apoptosis mediated by FOXO3a is suppressed. Interestingly, NO also affects other EGFR pathways in that

high levels of NO stimulate vessel endothelial growth factor which promotes angiogenesis, an important process in tumor growth.[79] Thus, NO and EGFR heterodimers may act synergistically in the progression of neoplastic lesions. Conversely, Jin *et al.* (2004) found breast cancer lesions expressing the active form of FOXO3a to have an increase in lymph node involvement and an increased invasiveness.[40] Therefore the function of FOXO3a may be multifaceted in the regulation of cellular signaling and neoplasia. Additionally the FOXO3 transcription factor targets upstream binding sites of the estrogen receptor alpha (ERα) B promoter region which stimulates ER production.[29] Over-expression of HER-2 causes FOXO3 to be phosphorylated and thus in an inactive form which cannot stimulate the B promoter region of ER. This interaction could, in part, explain the correlation between over-expression of HER-2 and ER negative aggressive tumor types (Figure 1.1).[29]

HER-2 EXPRESSION in CANCER

Using HER-2 antibodies, over-expression of HER-2 was found in 25-30% of human breast cancer patients,[59,90,91] and approximately 17-35% of canine mammary tumors (CMT)[57] (Figure 1.2, immunodetection of neu C18 (Santa Cruz Biotechnology, Santa Cruz, CA) with AEC (Biomedia, Foster City, CA) counter stained with Gills III hemotoxylin).[24] Dutra et al (2004) found that 35.4% of canine mammary tumors were positive for HER-2 and these were positively associated with nuclear pleomorphism, histologic grade and mitotic counts.[19] Additionally, c-erb-2 mRNA is overexpressed in approximately 74% of malignant CMT[1] and over-expression of HER-2/neu is associated with highly invasive ER-negative breast cancer in humans[14,25,59] and 57% of feline mammary carcinomas.[60] Moreover, cyclooxygenase-2 (COX-2) was expressed in 96% of

feline and 100% of canine invasive mammary carcinomas.[61] In bitches, an increased COX-2 expression was significantly correlated to HER-2 overexpression and to tumor dedifferentiation. In the canine, COX-2 may be important for mediating HER-2 induced mammary tumors.[60] In a retrospective study of 30 primary breast tumors with secondary distant metastasis, HER-2 expression increased in 24 of the 30 cases in the metastatic lesion.[83] Activation of HER-2 leads to a reinitiation of cell proliferation, disruption of tight junctions and loss of cell polarity in polarized epithlia,[65] all early events in tumorigenesis. Rungsipipat *et al.* (1999) suggested that HER-2 may be up-regulated in the initial development of canine mammary tumors (CMT) which may well make it a more accurate prognostic indicator early in the course of these cancers.[85]

Expression of HER-2 and EGFR are important in angiogenesis,[106] which is an important factor in tumor progression. Heregulin is a known ligand of HER-2 heterodimers,[84] and when added to cell populations over-expressing HER-2, vascular endothelial growth factor (VEGF) production is significantly increased.[105,106] Heterodimers including HER-2, particularly HER-2/HER-3 and HER-2/EGFR, upregulate VEGF and in turn angiogenesis. Yen *et al.*(2002) injected MCF-7 cells transfected to overexpress HER-2, into the fat pads of mice and the resulting tumor was immunohistochemically stained for HER-2, VEGF and CD31, (a blood vessel density marker). The lesions over-expressing HER-2 showed upregulation of VEGF and increased CD31 indicating an overall upregulation in angiogenesis.[105] Conversely, cells over-expressing *erbB-4* showed a decrease in VEGF indicating that the expression of different heterodimers provides regulation of angiogenesis by affecting differential expression of VEGF.[105]

Knowledge of the migration and invasiveness of a neoplastic lesion is an important prognostic and diagnostic tool. Thus it is of interest that HER-2 together with tumor growth factor beta (TGF β) appear sufficient to promote breast tumor cell migration and invasion to distant tissues.[87] However, upregulation of Erk appears to be required for maximal enhancement of these processes within tumorous tissues.[87] Interestingly, HER-2 expression may change in bone metastasis of breast cancer. Lorinez et al (2006) demonstrated a 17.4% positive HER-2 expression in primary breast cancer biopsies, with a decrease to only 10.5% positive HER-2 expression in bone, the most frequent metastatic site.[54]

Another important HER-2 expressing tumor is osteosarcoma (OSA) which shows striking similarity between human and dog. These tumors are primarily found in male patients with 50% of males more likely affected in both species.[101] Additionally the appendicular skeleton is affected in a majority of the cases, and the histopathology between humans and canines is similar. The most common site of metastasis in both species is the lung via vascular routes. There is a short disease free interval in both humans and canines with amputation alone, with an average of 2 years in humans and 6 months in the dog.[101] However human patients have disease onset younger in life (<20 yrs of age) whereas OSA in dogs generally affects patients in middle age (5-8yrs).[101] Currently there are few medical strategies with which to successfully treat OSA in both species. However new therapeutic strategies are aggressively being sought, though a lack of molecular understanding of these tumors has greatly inhibited the quest. Over-expression of HER-2 in OSA has been documented in several human and canine cases,[2,24,26,28,32,50,71,86](Figure 1.3, immunodetection of neu C18 (Santa Cruz

Biotechnology, Santa Cruz, CA) with AEC (Biomedia, Foster City, CA) counter stained with Gills III hemotoxylin), but the results for HER-2 experssion in OSA are controversial with some studies reporting up to 61% positive expression and others reporting little to no HER-2 expression.[5,92,100] Furthermore, seemingly contradictory results indicate that over-expression of HER-2 maybe a positive[2,26], negative[24], or neutral[71] prognostic indicator depending on the study parameters and methodologies utilized. However, Fellenberg et al (2004) found that risk assessment based on increased HER-2 gene expression, analyzed by real-time PCR, matched the histological findings in 94% of OSA biopsies.[22] Additionally human patients with HER-2 expression in primary OSA were more likely to have multiple metastases and shorter disease-free intervals compared to patients in whom HER-2 expression was not observed.[23]

HER-2 and CAV-1

Caveolin-1 (CAV-1) is a highly conserved gene[30] that has been implicated in a number of human disease processes including diabetes,[20] Alzheimer's[20] and breast cancer.[16] The caveolin proteins are a major component of caveolae, flask-like invaginations in the microstructure of the cell membrane that are enriched with cholesterol and lipids.[80] Additionally, caveolins interact with numerous signaling molecules including EGF, the Src tyrosine kinases, e-NOS, G-proteins and Ras.[53] CAV-1 is localized to a region on the human genome which has been shown to be commonly deleted in a number of tumors including breast cancer.[20] An overall loss of CAV-1 expression may enhance cell growth[48] while concurrently inhibiting cell death ultimately leading to a neoplasic lesion.[82] Interestingly, CAV-1 also carries a scaffolding domain and has been co-localized to the membrane with membrane bound ER□.[80,62] This

scaffolding domain of CAV-1, with the assistance of aromatic residues, directly inhibits EGFR. There are two mechanisms to possibly explain this result: one is that CAV-1 interaction stabilizes EGFR in the inactive form, the other possibility being that CAV-1 binding blocks receptor dimerization[15] the importance of which has been discussed previously. By blocking dimerization over-expression of CAV-1 ultimately leads to lower levels of HER-2 expression, which require dimerization for functionality. Estrogen has been shown to stimulate the production of CAV-1 causing the translocation of ERα to the cellular membrane.[80] Moreover, in the absence of estrogen ERα translocation to the cell membrane is dependent on CAV-1.[21] Once at the cell membrane ERα act much like other G-proteins and active SRC via several pathways including PLC, IP3 and PKC.[21,56] In breast cancer active SRC leads to activation of EGFRs like HER-2 via matrix metalloproteinase-2 and 9. Once activated the EGFR promote cell growth and survival via PI3-kinase and ERK (Figure 1).[21] Thus CAV-1 may play a role in regulating EGFRs in during cell growth and differentiation as well as promoting neoplastic transformation.

HER-2 and ER ALPHA

Estrogen is known to play a regulatory role in the normal development of the mammary gland.[90] Estrogens also influence many proto-oncogenes that are involved in tumor development[90] and mammary tumor growth.[97] Thus, ER is now the number one genetic prognostic factor in human breast cancer[6] with, high nuclear ER expression correlated with a lower histologic grade and thus longer survival times.[36,55,62,93] Moreover, Nieto *et al.* (2000) determined that CMT with a low level of nuclear ER expression had a higher incidence of distant metastasis during the followup period of 18

months.[68] Over-expression of HER-2 in breast cancer lesions is highly associated with a negative expression of ERα.[75] It is though that over-expression of HER-2 blocks ER transactivation and suppresses ER target genes resulting in a repression of genomic response by HER-2 on estrogen receptor.[75] Additionally deregulation of HER-2 is associated with ERα sequestration in the cytoplasm.[47,103] Interestingly in ER α positive MCF-7 cells that over-express HER-2 also show over-expression of metastatic tumor antigen 1 (MTA1). MTA1 is associated with aggressive behavior of several human cancers including breast cancer.[47] Additionally MTA1 is a co-repressor of nuclear ERα and has been co-localized to with ERα in the cytoplasm in MCF-7 cells that over-express HER-2. This cell phenotype shows increased metastatic behavior when injected into nude mice.[47] Another theory behind ERα sequestration in the cytoplasm is that ERK block the entry of ERα from entering the nuclear compartment.[103] Evidence in support of this interaction is that HER-2 over-expressing cells also have elevated levels of ERK, and blocking ERK allows ERα to re-enter the nuclear compartment.[103]

Recently several studies have examined the potential for cross talk between HER-2 and ERα in an effort to explain the common occurrence of tamoxifen resistance in breast cancer. Estrogen has both nuclear as well and cytoplasmic effects on cell survival and growth. Interestingly, estrogen mimics the ligand for HER-2 heterodimers.[58] Additionally, Shou et al 2004 showed that estrogen rapidly activates HER-2 as well as AKT, and the MAPK pathway.[89] Moreover, activation of the AKT pathway by estrogen requires HER-2/HER-3. The mechanism of which maybe the recruitment of PI3-kinase and the subsequent activation of AKT.[95] Thus in the absence of ERα it is likely that estrogen is able to stimulate cell growth and survival via an unconventional pathway of

stimulating cell membrane growth factors. In addition to direct stimulation of HER-2 by estrogen, ERα is sequestered in the cytoplasm of some HER-2 over-expressing breast cancer cell line as well as aggressive lesions. This phenomenon may not only limit nuclear stimulation of ERα but may also act as a target for membrane activation of estrogen, leading to down stream activation of HER-2 and subsequently the ERK pathway.[81] Briefly, ERα and HER-2 are associated with caveolin membrane rafts.[80] Estrogen stimulation of MCF-7 cells further stimulates the association of ER-2 and HER-2.[103] Additionally, when membrane associated ERα is activated by estrogen it leads to G-protein activation of matrix metalloproteinases (MMP) via Src.[81] Activation of MMP-2 and MMP-9 lead to the cellular release of heparin binding EGF, a ligand for the EGFRs[81] and activation of the ERK pathway.[81,103] The above mechanisms begin to demonstrate how ERα negative cells are stimulated by estrogen to enhance cell growth and survival as well as becoming anti-estrogen resistant.

In the past, the determination of prognosis in cancer has been based on histopathology and staging. While these modalities of diagnosis will not become obsolete, the use of genetic expression to assist in the determination of prognosis will become exceedingly important. As outlined above, HER-2 plays an important role in many different pathways intimately involved with cancer; many of which have been well documented while others remain to be elucidated. Understanding these complex molecular interactions is critical to further progress in the battle against devastating events such as mammary tumors and osteosarcomas in multiple species. The

identification of under- and over-expressed genes will continue to be an important diagnostic tool in oncology and will provide the groundwork for continued studies examining them as potential targets of adjunct therapy.

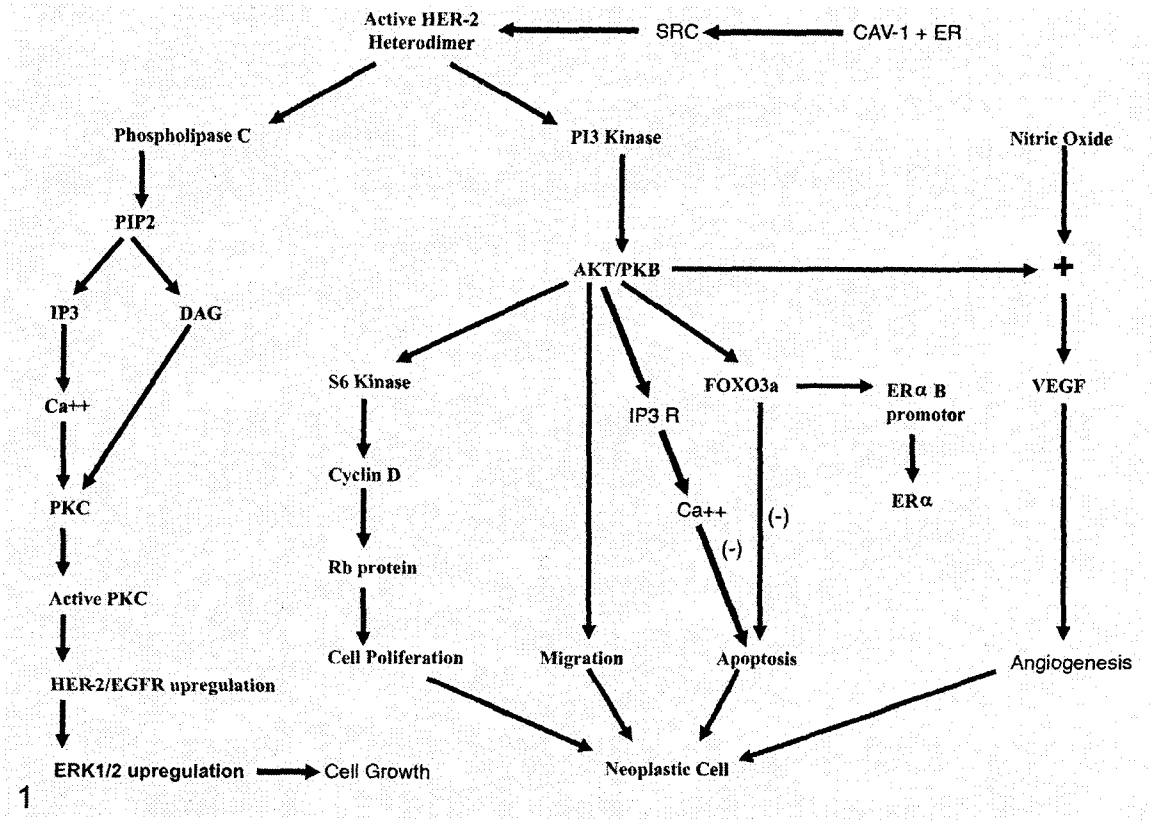

Fig. 1.1. Summary of the role HER-2 plays to prevent cell death and stimulate cell proliferation. The interaction of HER-2 with important cell signaling proteins increases the potential of a neoplastic lesion.

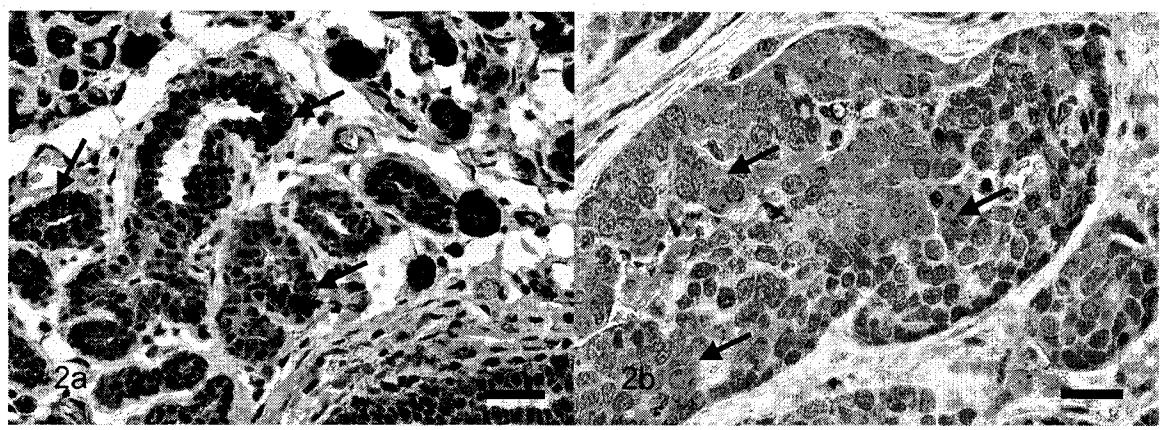

Fig. 1.2. Immunohistochemical staining of 2 canine mammary tumor sections. 2a represents a highly positive lesion 2b represents less intensive staining. Arrows indicate areas of increased stain intensity. The chromogen is AEC and Gill III hematoxylin the counter stain. bar = 40 μm.

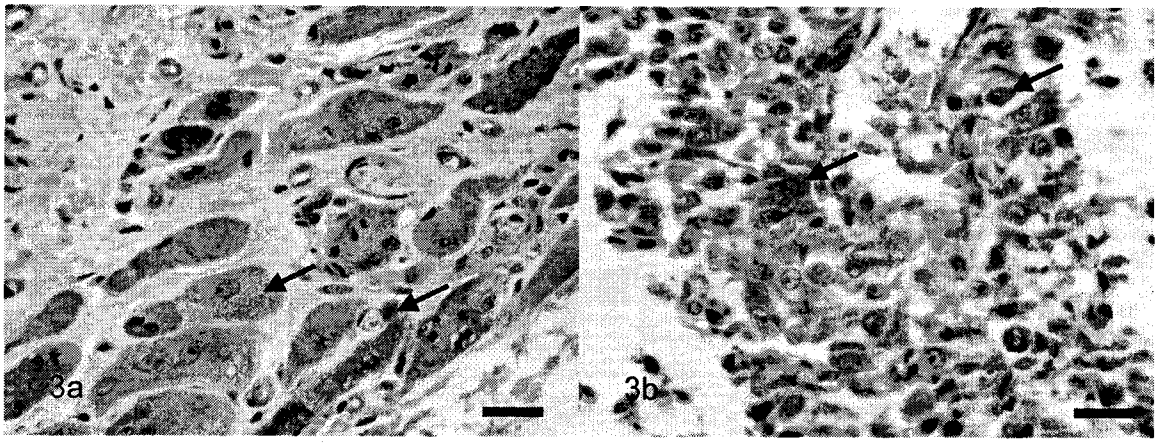

Fig. 1.3. Immunohistochemical staining of 2 canine osteosarcoma sections. Arrows indicate cells with increased stain intensity. The chromogen is AEC and Gill III hematoxylin the counter stain. bar = 40 μm. (Flint et al 2004)

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Chapter 2

Over expression of the *erbB-2* Proto-Oncogene in Canine Osteosarcoma Cell Lines and Tumors

INTRODUCTION

Osteosarcoma (OSA) is the most frequent highly malignant bone tumor in canine as well as human patients.[3,5,8] The use of canine OSA as a model of human OSA is compelling due to the fact that they bear the following striking resemblances: males are more commonly affected in both species; metaphyseal sites in appendicular bones are most often affected; the etiology is unknown and less than 10% of patients have documented metastasis at presentation. Over 90% of canine OSA show high grade histology with the metastatic rate more than 80% with amputation alone, primarily to the lung.[2,12,14] Research into the molecular mechanisms underlying canine OSA would provide necessary information toward better treatment of both canine and human patients.

ErbB-2 is a proto-oncogene that encodes human epidermal growth factor receptor 2 (HER-2). HER-2 is a 185 kD transmembrane glycoprotein of the tyrosine kinase family of receptors.[4] To date no known ligand has been found for HER-2; however, activation of HER-2 induces a cascade of mechanisms resulting in cell transformation and growth.[6] HER-2 has been extensively evaluated in both human and canine breast cancer. In both cases HER-2 over expression is correlated to poor prognosis.[1,11] In addition a correlation between HER-2/*erbB-2* over expression, poor prognosis and decreased survival in human OSA patients has been proposed by Gorlick et al 1999[5] and Onda et al 1996.[9] Therefore, HER-2/*erbB-2* expression maybe a useful prognostic indicator in canine OSA.

MATERIALS and METHODS

ErbB-2 expression levels were measured in mouse fibroblast cells, cell lines derived from canine osteogenic sarcomas and canine OSA samples, with the ultimate purpose of evaluating the potential utility of canines as models for human OSA as well as measuring erb*B*-2 gene expression as a diagnostic indicator in canine OSA. To accomplish this goal NIH/3T3 and B104-1-1 (NIH/3T3 cells engineered to over-express *erbB-2*),[7] both mouse fibroblast cell lines, and 7 osteogenic canine cell lines were utilized. OSA cell lines were grown in modified MEM (Cellgro, Herndon, VA) and 10% FBS (Hycolne, Logan, UT). NIH 3T3 and B104-1-1 cells were grown in DMEM (Gibco, Grand Island, NY), and 10% FBS (Hycolne, Logan, UT). All cells were maintained at 37° C in 5% CO2. OSA and control bone samples from the same individual were also obtained from ten different canine patients presenting to the Colorado State University Veterinary Teaching Hospital. Histopathology was performed on all samples to confirm the presence or absence of OSA. In addition samples were flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. Cell lines were detached from flasks with 0.25% trypsin and disrupted using QIAshredder (Qiagen, Valencia, CA). Total RNA was then isolated from the samples using RNeasy Mini Kit (Qiagen, Valencia, CA). RNA from OSA tissue and normal bone was isolated by homogenizing the tissue under liquid nitrogen, disrupting the homogenate in a QIAshredder (Qiagen, Valencia, CA), and collecting RNA via the RNeasy Mini Kit (Qiagen, Valencia, CA). One *u*g of total RNA was Dnase-treated and reverse transcribed (RT) using Invitrogen reagents (Invitrogen, Grand Island, NY). For each sample two PCR reactions were performed using GAPDH and erb*B*-2 primers. The sequences of primers utilized were as follows: GAPDH

forward 5'-GGC AAA TTC CAC GGC ACA GTC AAG GC-3', reverse 5'-CAG AGG GGC CGT CCA CGG TCT TCT GGG TGG-3'; erb*B*-2 forward 5'-ACC AGG CCC TGC TCC ATA GT-3' and reverse 5'-GTA CTC GGC ATT CCT CCA CG-3'. PCR reactions totaled 50 μL and were comprised of the following: 6 μL of cDNA, 1X PCR buffer (Sigma, St. Louis, MO), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.12 mM each forward and reverse primer, 1 unit of Platinum Taq (Invitrogen, Grand Island, NY), and PCR grade water to volume. Thermocycler parameters were 15 sec at 95°C, 30 sec at 55°C, 1 min at 72°C for 30 cycles. Each reaction included an initial denaturation at 95°C for 5 min and a final extension at 72°C for 2 min.

Real-time RT-PCR was preformed on all samples utilizing a Bio-Rad iCycler (Bio-Rad, Hercules, CA). For this assay, two PCR reactions per sample were performed using parameters as described above with the following addition: 1 μL Sybr Green (Molecular Probes, Eugene, OR 1:1000 of 10,000X stock), 1μL flourocein (Bio-Rad, Hercules, CA 10 nM). Following PCR amplification a melt curve was performed to confirm that nonspecific products were not being amplified.

Each real-time reaction was analyzed by selecting the amplification cycle where the PCR product of interest first crosses the threshold of baseline fluorescence (C_T). The threshold cycle (C_T) was further defined as the fractional cycle number at which the fluorescence reaches 10x the standard deviation of baseline. The relative expression of *erbB-2* in each sample was calculated as described in the ledges of each table.

Utilizing GADPH as a control for level of gene expression we compared the levels of *erbB-2* expression in the B104-1-1 and NIH/3T3. Real-time RT-PCR of the B104-1-1 cells shows that as expected they, expressed *erbB-2* 33.8 times that of NIH/3T3

cells (Table 2.1). These data are consistent with other data from our laboratory, which show real-time technology to be a more highly sensitive methodology to study gene transcription. *ErbB-2* expression analysis of the 7 osteogenic canine cell lines revealed that 6 of the 7 cell lines (86%) significantly over expressed *erbB-2* (Table 2.2), further confirming *erbB-2* over expression in a high percentage of canine OSA. Comparison of canine OSA to unaffected bone from the same patient was also performed in order to minimize the effects of interbreed variation in *erbB-2* expression patterns. When these tissues were evaluated with real-time RT-PCR, 4 of the 10 samples (40%) analyzed significantly over expressed *erbB-2* (Table 2.3).

Immunohistochemistry (IHC) was used to determine if HER-2 expression was correlated to real-time RT PCR *erbB-2* data in NIH/3T3, B104-1-1 and the 7 osteogenic canine cell lines, in addition 2 of the 10 clinic cases were also stained for HER-2. Cells were plated onto permanox chamber slides (Nalge Nunc, Naperville, IL) and incubated at 37° C in 5% CO_2 for 48 hours. Cells were then washed with PBS and slides were fixed for 10 min in 1% paraformaldhyde and allowed to air dry prior to staining. Fixed cell slides were rinsed with PBS and permeabilized with 0.1% Triton-X 100, 0.4% goat serum (Vector Laboratories, Burlingame, CA) for 20 min at 37° C. Cell slides were then incubated with HER-2 (Neu c18) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1:500 for 15 min at 37° C. Following PBS wash cell slides were incubated with a biotinylated anti rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at 37° C. The cell slides were then washed in PBS and incubated with Avidin Biotinylated horseradish peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA) for 30 min at 37° C. Following PBS wash cell slides

were reacted with 3-amino,9-ethyl-carbazole (AEC) chromogen (Biomedia, Foster City, CA) for 10 min at 37° C. Finally cell slides were counter stained with Gills III hemotoxylin and cover slipped. Tissue sections (4μm) were mounted on plus slides and deparaffinized using two xylene washes and rehydrated in graded ethanol. Sections were peroxidase-blocked using 3% hydrogen peroxide for 5min. Antigen retrieval on rehydrated sections was done in 1X Citra antigen retrieval (Bio Genex, San Ramon, CA). Sections were washed in PBS and blocked with 0.4% goat serum. Following the blocking step IHC proceeded as above.

Cells and sections stained for HER-2 were evaluated using 40X objective and scored as HER-2-positive or HER-2-negative compared to the mouse fibroblast cell lines. Consistent with our analysis of *erbB-2* gene transcription, the OSA cell lines showed differential HER-2 expression (Fig. 2.1-2.4). This differential expression of HER-2 closely followed the expression levels for the *erbB-2* real time data presented here. In addition, IHC HER-2 expression in the tumor sections also followed real-time RT PCR *erbB-2* expression (Fig. 2.5-2.6)

RESULTS and DISCUSSION

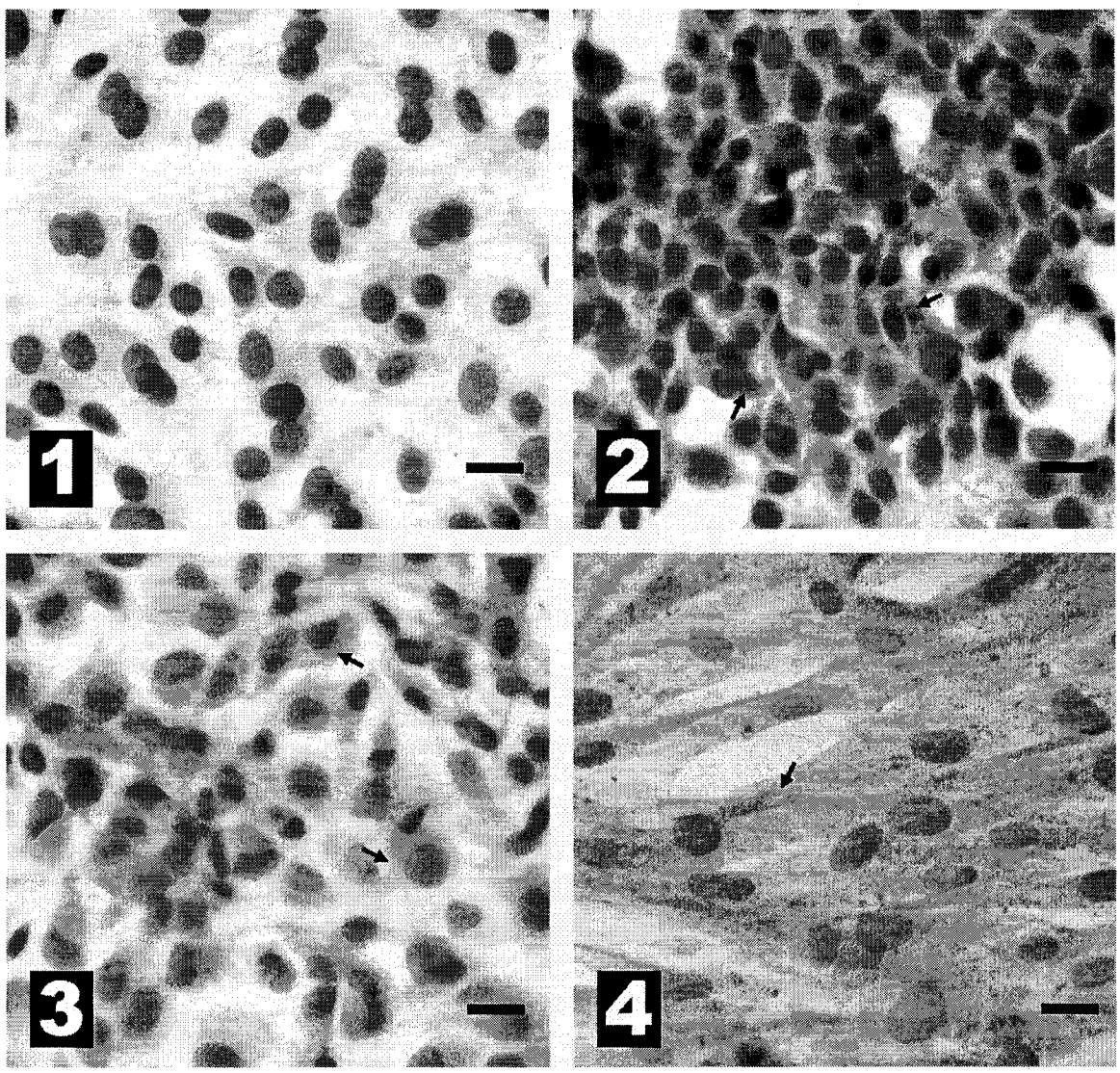
Of all the cancers in dogs, 10% of the caseload at the Colorado State University Veterinary Teaching hospital (oncology unit) are OSA. Moreover, OSA are extremely difficult to treat and represent one of the most common causes of death among small animal cancer patients.[3,12] Unfortunately traditional treatment strategies such as surgery, radiotherapy and chemotherapy have been unsuccessful in controlling these tumors.[2,12] The high prevalence of metastases and reoccurrence after surgery has prompted a search for methods of identifying patients that would benefit from early and aggressive, yet highly specific treatments for OSA. A firm and thorough understanding of the cellular events underlying this disease process would allow the potential development of effective and specific treatment regimens for both human and canine patients affected with OSA.

Over expression of *erbB-2* is associated with osteosarcoma as well as with other neoplastic transformation in a variety of human tumors including breast[11] and ovarian.[10] Moreover, over expression of *erbB-2* is strongly correlated with early pulmonary metastasis and poor survival rates for human patients with these cancers.[5,9,13] The data presented here support the hypothesis that over expression of *erbB-2* in OSA may be a useful prognostic marker for canine patients. Moreover, IHC analysis of HER-2 shows that indeed gene expression patterns for *erbB-2* are consistent with subsequent expression of functional HER-2, thus further implicating canine HER-2/*erbB-2* as a possible therapeutic target. To further substantiate the prognostic value of *erbB-2* over expression, longevity following diagnosis was collected for the ten clinical cases described in this study. Analysis of these data shows that over expression of *erbB-2* decreases longevity

by 115.5 days (Table 2.4). However given the limited number of dogs in our study this period of time was not statistically significant, yet the trend exists.

Initial results demonstrate that over expression of HER-2/*erbB-2* is detected in a significant percentage of canine OSA; thereby lending credence to the fact that HER-2/*erbB-2* may indeed be an important prognostic indicator and therapeutic target for the initial diagnosis and treatment of canine OSA. The results presented closely follow those of Gorlick et al 1999[5] and Onda et al 1996.[9] In those studies HER-2 over expression in human OSA patients was an indicator of poor prognosis.

ErbB-2 has been extensively studied for its involvement in a host of different tumors. In most cases over expression of the *erbB-2* gene correlates well with a poor prognosis, thus therapeutic approaches have been utilized to functionally inactivate it through the use of a monoclonal anti-*erbB-2* antibody (Herceptin™). Based on our current data its plausible to hypothesize that a similar approach could be utilized in the dog, thus resulting in improved treatments for dogs afflicted with OSA.



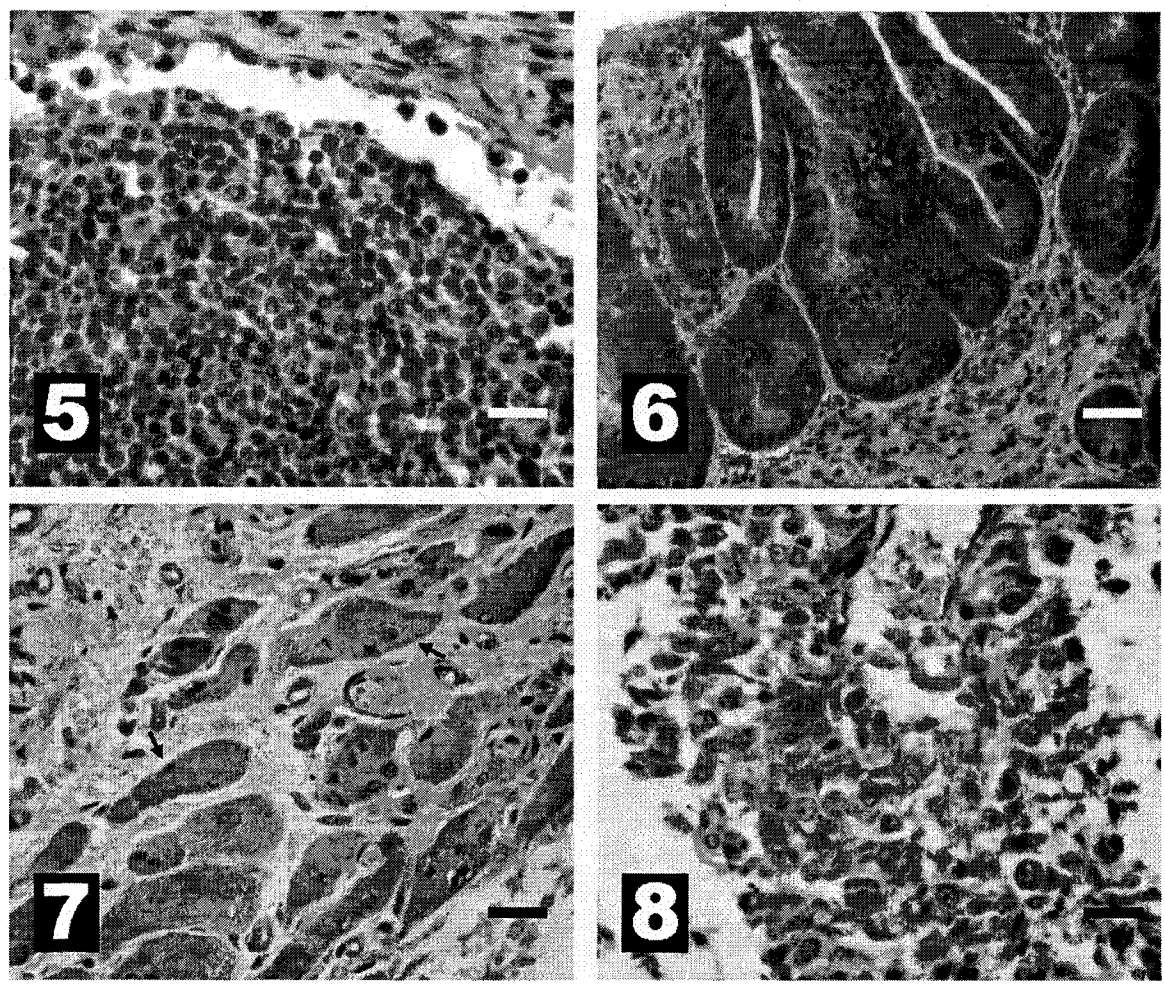


Table 2.1. Real-time RT-PCR measuring expression of *erbB-2* in NIH 3T3 and B104-1-1 (NIH 3T3 cells engineered to over express *erbB-2*) cell lines.

Cell Line [a]	ΔCT [b]	Relative Expression [c]
NIH 3T3	10.986	
B104-1-1	5.905	33.848

[a] All samples run in triplicate.

[b] ΔCT calculated as the (GOI CT)-(HKG CT).

[c] Relative expression calculated as $2^{\wedge -((\text{tumor } \Delta CT)-(\text{normal } \Delta CT))}$.

Table 2.2. Expression of *erbB-2* in canine OSA cell lines compared to average of normal bone samples.

Cell Line # [a]	ΔCT [b]	Relative Expression [c,d]	*erbB-2* Positive [e]
OSA-CL1	11.067	3.986	Y
OSA-CL2	13.367	0.809	N
OSA-CL3	11.000	4.175	Y
OSA-CL4	11.833	2.343	Y
OSA-CL5	11.100	3.895	Y
OSA-CL6	11.400	3.164	Y
OSA-CL7	11.367	3.238	Y

[a] All samples run in triplicate.

[b] ΔCT calculated as the (GOI CT)-(HKG CT).

[c] Relative expression calculated as $2^{-((\text{tumor } \Delta CT)-(\text{normal } \Delta CT))}$.

[d] Average of 10 normal bone samples was used as comparison. ΔCT = 13.062 ± 0.849.

[e] *erbB-2* positive if relative expression is 2 or more.

Table 2.3. Real-time RT-PCR evaluating the expression of *erbB-2* in canine OSA compared to normal bone, within the same animal, and survival time.

Case #	Sample [a]	ΔCT [b]	Relative Expression [c]	*erbB-2* Positive [d]	Survival Post Amputation (d)
1	OSA Tumor	12.277	1.666	N	927.00
	Normal Bone	13.013			
2	OSA Tumor	12.651	0.187	N	870.00
	Normal Bone	10.231			
3	OSA Tumor	11.168	5.602	Y	438.00
	Normal Bone	13.654			
4	OSA Tumor	11.120	6.712	Y	770.00
	Normal Bone	13.866			
5	OSA Tumor	15.282	1.421	N	434.00
	Normal Bone	15.790			
6 [e]	OSA Tumor	10.595	0.529	N	45.00
	Normal Bone	9.677			
7	OSA Tumor	11.004	0.462	N	155.00
	Normal Bone	9.890			
8	OSA Tumor	11.557	3.600	Y	37.00
	Normal Bone	13.405			
9	OSA Tumor	13.217	32.297	Y	241.00
	Normal Bone	18.230			
10	OSA Tumor	14.024	0.446	N	49.00
	Normal Bone	12.860			

[a] All samples done in triplicate.

[b] ΔCT calculated as the (GOI CT)-(HKG CT).

[c] Relative expression calculated as $2^{\wedge -((\text{tumor } \Delta CT)-(\text{normal } \Delta CT))}$.

[d] *erbB-2* positive if relative expression is 2 or more.

[e] Data point removed from survival data due to surgical complications.

Table 2.4. Comparison of *erbB-2* positive dog survival to *erbB-2* negative dog survival, post amputation. Dogs negative for *erbB-2* survived 115 d longer.

***erbB-2* status**	**Total Cases** [a]	**Mean Days of Survival Post Amputation (d)** [b]
Cases *erbB-2* Positive	4	**371.5**
Cases *erbB-2* negative	5	**487**

[a] Of 9 cases 7 died of lung metastasis and 2 died of other causes.

[b] Results not significant due to low sample number.

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Chapter 3

Prognostic Significance of HER-2, CAV-1, and ER alpha Expression in Canine Mammary Tumor

INTRODUCTION

The most common neoplasm encountered in canine medicine, accounting for approximately 50% of the total diagnosed tumors,[2,37] is mammary cancer.[39] Similarly, breast cancer accounts for approximately 40% of tumors diagnosed in human medicine.[43] Studies have shown an increased incidence of mammary neoplasia in Toy and Miniature Poodles, English Springer, Brittany and Cocker Spaniels, English Setters, Pointers, German Shepherds, Maltese, Yorkshire Terriers, and Dachshunds[45] indicating a genetic/familial component associated with canine mammary tumors (CMT) much like what is known to occur in human breast cancer. The percentage of malignant CMT varies widely from 11% to 100%[4] and there is a high incidence of metastasis and reoccurrence usually within 1 year of surgery.[2,45] Unfortunately, dogs with malignant mammary neoplasia have a mean survival time of 4-17 months with a 2-year survival of 25-40%.[7] Much like CMT, 79% of breast cancer in women is invasive[42] and breast cancer diagnosed in women with regional involvement has a 5 yr survival of 70% while lesions with distant metastasis drop this percentage to 15-20%.[42] Currently, in veterinary medicine, surgical removal is the most routine modality of treatment.[45] In some cases adjuvant therapy is also utilized, however, no agreement exists as to the best modality for treatment nor for the use of additional therapies.[45] This lack of knowledge of the underlying molecular characteristics of these tumors has prompted an interest in identifying the genetic aspects associated with CMT[2,37] in an effort to tailor therapy to tumor dynamics for the best prognosis.[37] Additionally, adjuvant therapies could be developed, much like Herceptin™ used in human breast cancer therapy, to better treat

dogs with aggressive tumors. Moreover, as the similarities between canine and human mammary cancer are elucidated the dog may prove to be a valuable model for human breast cancer molecular and pharmaceutical research.

Caveolin-1 (CAV-1) is a highly conserved gene[15] that has been implicated in a number of human disease processes including diabetes[11], Alzheimer's[11] and breast cancer.[10] CAV-1 is localized to a region on the human genome which is commonly deleted in a number of tumors including breast cancer.[11] The caveolin proteins are a major component of caveolae, flask-like invaginations in the microstructure of the cell membrane that are enriched with cholesterol and lipid.[30] Caveolins interact with numerous signaling molecules including epidermal growth factors, the Src tyrosine kinases, e-NOS, G-proteins and Ras.[18] CAV-1 also carries a scaffolding domain, underscoring its downstream influence on cell signaling, and has been co-localized with membrane-bound estrogen receptor alpha (ER□).[30] CAV-1 can bind and inactivate its associated signaling molecules slowing cell growth, which was demonstrated by Gabaiti et al (2001) as a concomitant increase in the expression of CAV-1 with NIH 3T3 cell confluency.[13] In this same study, nude mice injected with NIH3T3 cells containing an antisense CAV-1 developed tumors within 1-2 weeks post injection, however mice injected with parental NIH 3T3 cells showed no tumor development. Moreover, increased CAV-1 expression decreases activation of Erk, which is critical to G1-S phase transition and cell division, thus resulting in a cell cycle arrest and limitation of cell growth.[1,12] Mammary tumors have been shown to over-express Erk resulting in CAV-1/ER□ disassociation allowing an estrogen-ER□ interaction which tends to decrease tumor cell apoptosis.[30] CAV-1 haploinsufficiency, which would in effect decrease ER alpha

association, is known to result in partial transformation of human breast epithelial cells.[56] Further evidence implicating CAV-1 as a "tumor susceptibility gene" was demonstrated using CAV-1 knockout mice which showed a dramatic increase in ductal growth and an increase in the number of tumors within the breast tissues.[51] In another study, CAV-1 null mice had an increase in response to tumorigenic stimuli thus providing evidence that CAV-1 plays a role in down-regulating cell growth when normally expressed.[15] Additionally, CAV-1 has been shown to be down-regulated in transformed human breast cancer cell lines and in human breast cancer tissues,[8,17] which appears to limit invasion and anchorage independent growth of these cells,[11] Interestingly, 16% of human breast cancer cases show aberrant expression of CAV-1.[14,52] A study of 55 primary invasive breast carcinomas showed that 25% of these lesions had dysregulation of CAV-1 compared with a 7.3% CAV-1 dysregulation of the paired normal samples.[8] Similarly, Sagara et al (2004) found that CAV-1 was down-regulated in human breast cancer and concluded that CAV-1 may play a role in tumorigenesis although there was no correlation to survival time with a recheck time limited to 3 years.[38] In a study of human inflammatory breast cancer (IBC) CAV-1 was expressed in 41.4% of IBC compared to 15.6% of non-IBC lesions.[49] In conclusion, an overall loss of CAV-1 expression appears to enhance cell growth[17] while concurrently inhibiting cell death ultimately leading to cancerous lesions in non-IBC lesions,[33] while over expression is reported in IBC-type lesions.[49] Moreover, the stage and morphology of breast cancer lesions may be responsible for the differential expression of CAV-1.[8]

Estrogen plays a regulatory role in the normal development of the mammary gland.[44] Estrogens also influence many proto-oncogenes and are involved in tumor

development[44] and mammary tumor growth.[48] Thus, ERα has been elevated to the number one genetic prognostic factor in human breast cancer.[3] ERα has been shown to regulate tumor responsiveness to estrogen, thus enabling treatment with estrogen blocking agents such as tamoxifin.[26] Recently several studies have identified a link between ERα cytoplamsic sequestration and HER-2 deregulation.[54] Moreover, the mechanism of tamoxifin resistance, that is ERα non-responsive growth, has been linked to estrogen binding of HER-2. The unique mechanism by which estrogen bypasses ERα binding to HER-2 may provide a tool for prognosis and a therapeutic target. Additionally, membrane bound ERα via activation of MMP-2 and MMP-9 and subsequent release of heparin binding EGF stimulates increased activity of EGFR dimers. In human breast cancer, high nuclear ERα expression has been correlated with a lower histologic grade and thus longer survival times.[16,20,27,45] Additionally, a study of 228 CMT showed that ERα positive lesions were most frequently benign lesions wereas ERα negative lesions were mostly malignant.[21] However, Nieto *et al* (2000) determined that CMT with a low level of nuclear ERα expression had a higher incidence of distant metastasis during the follow up period of 18 months.[28] Additionally, seven of those cases were lymph node-positive with these lesions being ERα negative in both the primary and metastatic sites.[21]

HER-2 (neu, *erb-B-2*) is a proto-oncogene that encodes for the epidermal growth factor receptor 2, a transmembrane protein with intrinsic tyrosine kinase activity.[34] HER-2 was initially found to mediate the proliferation and differentiation of normal epithelial cells[9,29] and is expressed in the development of normal breast tissue with HER-2 playing a role in lobuloaveolar differentiation.[29] Receptor activation results in a biological

increase in proliferation, morphological differentiation, cell motility and invasion.[29,37,47] Altered expression of HER-2 has been identified in 25-30% of human breast cancers,[22,40,41] approximately 17% of CMT[21] and 57% of feline mammary carcinomas.[25] Interestingly, feline mammary tumors are characterized by their aggressive behavior,[19] suggesting HER-2 expression is associated with more aggressive tumors. Additionally, over expression of HER-2 is associated with highly invasive ERα negative breast cancer in humans.[22,36] In a retrospective study of 30 primary breast tumors with secondary distant metastasis, HER-2 expression increased in 80% of metastatic lesions.[35] Rungsipipat et al (1999) suggest that HER-2 may be unregulated in the initial development of CMT which may well make it a good prognostic indicator in the early diagnosis of these cancers.[37] In addition, mutation of HER-2 down regulates CAV-1 in mammary tumors of transgenic mice.[11] Herceptin™, a commercially available HER-2 antibody, blocks HER-2 signaling, inducing G1 arrest, preventing angiogenesis and thus limiting tumor growth.[11,17] The complex interplay of HER-2, CAV-1, and ERα is only part of the genetic mal-expression that leads to tumor development, suggesting that development of therapeutics targeting multiple genes may be beneficial in treating aggressive cancers.

This study set out to determine if a pattern of multiple genetic dysregulation will emerge that is correlated with tumor morphology and metastatic potential of CMT. Specifically, differences in protein expression of CAV-1, and HER-2 are evaluated. These two genes have recently been implicated in critical functions within mammary tumor cells as outlined above. The expression of these proteins will be compared to tumor morphology, histological diagnosis and survival and evaluate correlations that may

lead to a better understanding of tumor dynamics and the adjustment of patient treatment for the best prognosis.

MATERIALS and METHODS

Tumor Specimens

156 CMT that were originally fixed in 10% buffered formalin and paraffin embedded were selected from the Colorado State University Diagnostic Laboratory archives dating from 5/31/01 to 7/30/03 (signalment Tables 1,2,4,6). For this study, two independent veterinary pathologists evaluated each case for histopathological diagnosis and morphologic description; in cases where the pathologists differed in opinion, the case was reviewed. Determination of histopathologic diagnosis followed Dr. Benjamin et al (1999) classification scheme.[4] All microcopy in this study was performed on an Olympus BX4, and all photomicrographs were taken using a Zeiss 200M inverted microscope with an ORCA-ER cooled interline charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). In addition to morphologic diagnosis, invasion, growth pattern, schirrhous reaction, percent necrosis, mitotic rate, degree of anaplasia, degree and type of inflammation were evaluated.

Immunohistochemistry

The antibodies utilized in this study and their sources are as follows: anti CAV-1 rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA), anti HER-2 rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA), anti ER IgG mouse monoclonal 1d5 (Dako, Carpinteria, CA). Serial sections from formalin fixed paraffin embedded blocks were cut 5m thick and attached to Plus TM slides (Fisher Scientific).

The slides were deparaffinized in xylene, rehydrated in descending EtOH concentrations, and blocked for endogenous peroxidase in 3% H_2O_2. Slides immunolabled with CAV-1 and HER-2 were pretreated in citrate buffer for antigen retrieval (BioGenex, San Ramon, CA), by microwaving slides for 2.5 min at 700W and then 10 min at 200W. Slides immunolabled for ER were cooked in citrate buffer in a pressure cooker for 25 min at 98°C. Slides were cooled for 15 min, washed under DI water for 10 min and finally placed in PBS for 5 min. All slides to be labeled were incubated with normal serum (Vector Elite, Vector Laboratories, Burlingame, CA) diluted in PBS at 37°C for 20 min to prevent nonspecific binding. Following blocking CAV-1 and HER-2 slides were washed for 5 min in PBS. Each primary antibody was diluted in PBS to the following concentrations: CAV-1, 1:100 and HER-2, 1:500. Each antibody was added to the slides to cover the tissue. The slides were incubated at 37°C for 15 min using CAV-1 and HER-2 on a Hi Pro slide incubator (Thermo Electron Corp, Waltham, MA). Following antibody labeling, CAV-1 and HER-2 slides were washed for 5 min in PBS. CAV-1 and HER-2 slides were incubated with a goat-anti-rabbit biotinylated secondary antibody (Vector Elite, Vector Laboratories, Burlingame, CA), for 30 min at 37°C. Following primary antibody labeling CAV-1 and HER-2 slides were washed with PBS. CAV-1 and HER-2 slides were then incubated for 30 min at 37°C with Avidin Biotinylated horseradish peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA). Following PBS (CAV-1 and HER-2/neu) washes all slides were immunodetected with AEC (Biomedia, Foster City, CA) for 10 min at 37°C and washed in DI water. Hematoxylin stain (Gills III Formulation #1, Fisher Scientific) was applied to all slides for 25 sec as a nuclear counter stain. Slides were then washed for 15 min under DI water and coverslipped.

Staining procedures were performed on 40 slides simultaneously 3x/ day to reduce variability. Capillary endothelial cells were used as positive controls for CAV-1 and human breast carcinoma was utilized as a positive control for HER-2. The primary antibody was omitted for negative controls.

ERα immunolabeled slides were stained using the Cadenza slide system (Thermo Electron Corp, Waltham, MA) as follows. Following citrate antigen retrieval and cooling slides were loaded into the cadenza slide holders and washed with TBS 0.05% Tween. Slides were blocked with normal serum (Vector Elite, Vector Laboratories Burlingame, CA) for 20 min at 37°C. ERα antibody (Dako, Carpinteria, CA) was diluted 1:50 and slides were incubated for 120 min at 37°C. Slides were washed with TBS/Tween and then incubated with anti-mouse biotinylated secondary antibiody (Vector Elite, Vector Laboratories, Burlingame, CA) for 30 min at 37°C. Slides were again washed with TBS/Tween and incubated with ABC for 30 min at 37°C and immunodetected with 2 applications of AEC (Biomedia, Foster City, CA) for 10 min at 37°C. Following immunodetection slides were washed with DI water and counterstained with hematoxylin and coverslipped as above. Positive controls were human breast carcinoma and were run with each staining group. Negative controls utilized a mouse IgG class 1 (Sigma-Aldrich, St Louis, MO) in place of the ERα antibody.

CAV-1 and HER-2 were evaluated semiquantitatively by 2 independent histopathologists. The sections were scored as follows: staining intensity [1/weakly positive, 2/moderately positive, 3/strongly positive]; percentage of cells staining within the tumor [0/0-6%, 1/7-25%, 2/26-50%, 3/51-75% 4/76-100%]. These two values were multiplied together creating a composite score.[10] The average of both evaluators'

composite score was then converted to a 1-3 score [1/0-4, 2/5-8, 3/9-12]. The 1-3 scoring was used in the statistical analysis. When extreme differences were observed between evaluators that slide was observed by both evaluators using a dual head microscope. Evaluation of ERα was again preformed by two independent microscopists. The lesion was evaluated as positive or negative for staining in both the nuclear and cytoplasmic compartment.

Histopathology

All sections were stained with H&E and evaluated for histopathology by a Board Certified Veterinary Pathologist. Only one side per case was evaluated to determine the following histologic parameters: growth pattern [ductual, papillary, solid, mixed], invasion [expansile, local, regional, nodal, vascular], schirrhous reaction [1/minimal, 2/moderate, 3/marked], percent necrosis [1-100%], mitotic rate [number per 10 high power fields, chosen from areas in the mass with mitotic activity], degree of anaplasia [1/mild, 2/moderate, 3/marked], degree of inflammation [1/mild, 2/moderate, 3/marked], and inflammatory cell type. Additionally all sections were given one of the following morphologic diagnosis per Benjamin et al (1999): mammary lobular lesions: lobular hyperplasia, adenoma, benign mixed, adenocarcinoma; and duct Lesions: ductular hyperplasia, duct papilloma, ductular papillary carcinoma, ductular carcinoma, anaplastic carcinoma.[4] If warranted, each tumor classification was further subdivided into simple or complex. Finally, as only one slide was evaluated from each case, the primary report was reviewed for further information regarding lymph node involvement, of which there were 10 samples that included a lymph node for evaluation.

Patient Outcome

In order to evaluate if CAV-1, HER-2 or ERα could be correlated with prognosis we surveyed the referring veterinarians for each case. This survey asked for treatment modality, remission or reoccurrence and information on type number and location of metastasis. Additionally, general information in regards to the patient's general health was requested: specifically, the body condition score of the patient (1 = thin, 5 = obese), status of intact or neutered, date of alteration, whether or not the patient was a breeding female and the number of litters and finally if the patient had any concurrent health problems. Additionally, a follow-up survey was sent approximately 18 mos following the first survey to the referring veterinarians, to ascertain the life status of the patient. At this time the initial survey was sent to those veterinarians that did not respond to the first mailing. Of the 156 cases, 117 surveys were returned with usable information (75%).

Statistical analysis

A preliminary evaluation of significant splits in the data was preformed using JMP (SAS Institute Inc, Cary, NC). This program evaluates natural data splits. With the results from this method, we were able to better tailor the statistical analysis. To evaluate the significance of HER-2, CAV-1 and ERα at predicting morphologic diagnosis, benign/malignant status, and patient outcome logistic regression was used. For those predictor categories that were not binomial SAS uses "proc genmod" to systematically evaluate a multinomial data set in binomial fashion. All statistical analysis was performed using SAS (SAS Institute Inc, Cary, NC). P values ≤ 0.05 were considered significant.

RESULTS

Of the 156 cases chosen, 16 were removed from the data set [1 insufficient tissue, 1 diffuse lobular hyperplasia, 1 mammary connective tissue, 11 insufficient lesion present in section, 1 soft tissue sarcoma, 1 fibroadenoma] leaving a data set of 140. There were 44 breeds represented with 89% (121/136) being purebred dogs. Beagles are over represented in this study as we utilized cases from a Collaborative Radiological Health Laboratory Beagle colony. Additionally, Cocker Spaniel, German Shepherd, Husky, Labrador Retriever had increased representation (Table 3.1). This correlates with other studies in which these breeds have been shown to have an increased incidence of mammary tumors.[45] The age of the patients in this study ranged from 1-17 yrs of age, averaging 9.1 yrs of age (Table 3.2). Patients 6 yrs of age and under were diagnosed with aggressive tumor types less frequently than those patients over the age of 6 yrs (Table 3.3). Neither HER-2, CAV-1 scores nor ERα status showed predilection to age of event. The body condition score (BCS) of the patients ranged from 2-5 (averaging 3.1) (Table 3.4) and had little value in predicting morphologic diagnosis (Table 3.5). Finally, intact females represented 57% of this population (Table 3.6).

Morphologic Diagnosis

Morphologic diagnosis of these tumors is presented in Table 3.7, the predominating diagnoses being adenocarcinoma, adenoma and benign mixed tumors. In order to better the statistical model, tumor types with the same behavior were combined. Thus adenocarcinoma simple and complex, are grouped as adenocarcinoma; adenoma simple and complex are grouped as adenoma; ductular carcinoma and ductular papillary

carcinoma are grouped as ductular carcinoma and ductular hyperplasia, lobular hyperplasia and ductular papiloma are grouped as other benign classifications. Morphologic diagnosis was distributed evenly between the females in the study, with two exceptions, intact females had an increased number of adenomas and anaplastic carcinomas while neutered females had increased numbers of adenocarcinomas (Table 3.8). Neutered males were divided one each in the adenocarcinoma and benign mixed diagnosis.

Growth pattern was recorded in 137 of the cases, as one of the following: ductular (66% or 90/137), papillary (18% or 25/137), and solid (16% or 22/137) (Table 3.9). Invasion was observed as one of the following: expansile (49% or 67/137), local (24% or 33/137), regional (9% or 13/137), vascular (17% or 23/137), nodal (4% or 6/137), and combinations of the above (Table 3.10). Schirrhous reaction scores were 0-3 (0-none, 1-mild, 3-severe) and was graded 2-3 in 60% (84/140) of the cases. In 82% (114/139) of the cases the percent necrosis of the lesion was 30% or less. Degree of anaplasia scores were 0-3 (0-none, 1-mild, 3-severe) with 70% (98/140) of the cases scored as 2-3. 54% (75/140) of the lesions had mitotic figures numbering 3 or fewer. In 69% (95/138) of the cases the degree of inflammation was 1-2. A primarily lymphocytic component was observed in 56% (72/128) of the cases however, nearly all of the cases had some lymphocytic involvement indicating a degree of chronicity to the lesion while, 21% (27/128) of the lesions had neutrophil involvement.

Survival Time and Time to Reoccurrence

Survival time and time to reoccurrence were part of the referring veterinarian survey. Of the 144 cases originally chosen, 94 (67%) of the referring veterinarians

returned survey results. 48 of the cases reported survival data of those 15 cases having died or euthanized due to neoplasia ranging from 9-2402d. Thirty-one of the referring veterinarians reported a reoccurring lesion with a time interval of 23-1709d. Of the 6 veterinarians reporting reoccurance of mammary tumor 4 had the same histomorphology (Table 3.11). The median time to reoccurrence was decreased by 109d in the HER-2 over-expressing group (score 2-3) (Figure 3.1). Moreover median survival time was decreased by 276d in the HER-2 over-expressing group (Figure 3.2). Additionally the CAV-1 over-expressing group (score 2-3) had a decrease in median time to reoccurrence of 236d (Figure 3.3) and a decrease in median survival time of 292d (Figure 3.4). However due to limited numbers of survival and reoccurrence data the above trends in the data set did not have statistical significance ($p \leq 0.05$).

Time from age of alteration to event was determined from the referring veterinarian survey in 48 cases. In 29 of these cases, the patient was spayed less than twelve months prior to the disease event (Table 3.12). In 38% (11/29) of these patients an aggressive tumor type was diagnosed (Table 3.13). Additionally 25% (23/93) of the patients in the study were breeding females. In 57% (13/23) of these patients a more aggressive tumor type was diagnosed, compared to 45% (32/71) of non-breeding females (Table 3.14).

Immunohistochemistry

HER-2 was examined for extent (1-4) and intensity (1-3) of staining and these numbers were combined to score the sample (1-3) (Figures 3.5 a-f). In 38% of the samples (52/137) HER-2 was scored a 1, 38.6% (53/137) scored a 2, and 23.4% (32/137)

of the samples scored a 3. Though 62% (85/137) of the lesions showed an over expression (score 2-3) of HER-2 (Table 3.15).

HER-2 score was not correlated with breed or age. Interestingly, HER-2 scores when the patient had a BCS of 3 were higher than when the patient had a BCS of 4. When malignant status of the lesion was compared to HER-2 score 63.5% (33/52) score 1, 49% (26/53) score 2 and 43.8% (14/32) score 3 (Table 3.16, Figure 3.6 (p=.08). Logistic regression models show the same decreasing trend, in that as HER-2 score decreases, the percent of malignant lesions increase. However when lesions that over-express HER-2 (score 2-3) are compared to lesions that have no expression of HER-2 (score 1) the percentage of malignant lesions is 55% (40/77) compared to 45% (33/77) (Figure 3.6).

Additionally, morphologic diagnosis was compared to HER-2 score. The percentage of lesions that were diagnosed as adenocarcinoma at each HER-2 score were 38.8% (21/54) (score 1), 30.5% (33/108) (score 2/3) indicating a relative increase in the percent of malignant lesions as HER-2 expression decreases. Lesions diagnosed as adenoma showed an increasing HER-2 score trend: 29.6% (8/27) (score 1) and 35.2% (19/54) (score 2/3). There was no difference in the percentage of benign mixed lesions between high and low ER-2 scores 33.3% (10/30) (score 1), 33.3% (20/60) (score 2/3). Ductular carcinoma had the following percentages compared to HER-2 72.7% (8/11) (score 1), 13.6% (3/22) (score 2/3). Lesions classified as other benign had the following percentages compared to HER-2 25% (2/8) (score 1), 37.5% (6/16) (score 2/3). Lesions classified as anaplastic carcinoma had the percentages compared to HER-2 50% (4/8)

(score 1), 25% (4/16) (score 2/3) (Table 3.17, Figure 3.7). Though these trends exist in the data when they were evaluated using logistic regression none were significant ($p>.05$)

CAV-1 was examined for extent (1-4) and intensity (1-3) of staining and these numbers were combined to score the sample (1-3) (Figure 3.8 a-f). In 70% (98/139) of the samples CAV-1 was scored 2-3, and in 31% (44/139) CAV-1 was scored a 1 (Table 3.18). CAV-1 score was not correlated with breed, age or BCS.

The malignant status of the lesion was not different among CAV-1 scores ($p>.05$): 56.1 % (23/41) score 1, 50% (26/52) score 2, and 54.5 % (24/44) score 3 (Table 3.16, Figure 3.9).

We also compared CAV-1 scores to the morphologic diagnosis of the lesion. In this comparison the number of adenocarcinoma lesions increased with an increasing CAV-1 score as did benign mixed lesions though they were not significant when used in a logistic regression ($p>.40$). The number of adenoma and anaplastic carcinomas did not increase or decrease in the face of CAV-1 score ($p>.80$). The number of ductular carcinomas decreased with increase CAV-1 score with a significance of $p=.18$. Additionally, the number of lesions diagnosed as other benign decreased with an increasing CAV-1 score ($p<.05$) (Table 3.17, Figure 3.10).

99 lesions were evaluated for ERα status in both the nucleus and in the cytoplasm (Figure 3.11 a-e). Of these lesions 28 (28%) and 22 (22%) had positive immunohistochemical staining in the cytoplasm and nucleus respectively (Table 3.19). ERα status was not correlated to age, breed, or BCS.

There was no difference in the status of ERα in the nucleus of the cells of the lesions between benign and malignant, 29% (11/38) and 28% (11/39), respectively (Table

3.16). When ERα was observed in the cytoplasm of the cell those lesions tended to be benign. Only 8 of the 28 (29%) of the positive lesions were malignant. When the cytoplasm was negative for ERα, 42 of the 71 lesions were malignant (Table 3.16). ERα status did not predict morphologic diagnosis in our study (Table 3.17) however, a majority of the adenocarcinoma lesions (14/16) and the ductular carcinoma (6/7) were ERα negative in the nucleus. Additionally, ERα was positive in nucleus of a lower number of both adenoma (10/31) and benign mixed (8/19) lesions. However, when the lesion was positive for ERα in the cytoplasm the lesion was more likely to be benign than if the lesion is negative for ERα with an odds ratio of 0.281 (p=0.0091).

There was no difference in time to reoccurrence for patients with ERα positive cytoplasm compared to those who were negative, however, those patients that had ERα positive nuclear lesions had a median survival time of 26 days less than those who were negative. Survival data for the ERα group were very limited so it is not reported here.

Finally, using the JMP program from SAS we evaluated the interactions of the three proteins of interest. This program looks at each data set and identifies significant splits in the data. Additionally, constraints can be made in order to see interactions between subsets of a data set. With this program we were unable to identify any significant interactions between our proteins of interest.

DISCUSSION

This study sampled tumors from 44 different canine breeds. Of these, 5 breeds were over represented in the population: Cocker Spaniel, German Shepherd, Husky, Labrador Retriever, and the Beagle (though these numbers are skewed due to the

inclusion of a set of Beagle colony tumors). Similar results for overrepresentation of these breeds have been shown in other studies.[45,53] Thus it may be prudent to inform owners of the above breeds about the risk factors of CMT and how to evaluate their dogs for suspect lesions.

Our study showed neutered females were less likely to be diagnosed with any type of breast cancer when compared to intact females (Table 3.8). However, it is apparent from Table 14 that time from the disease event to the time of neuter is important.[7] Initially, neutered females were more likely to be diagnosed with an adenocarcinoma. However if the population of females neutered less than 12 mo prior to the disease event are removed, the number of adenocarcinoma diagnosis drops well below those that were not neutered, and balances the percentage of more aggressive tumor types between the two groups. Additionally, patients that were breeding females were diagnosed with a more aggressive tumor type than those that were non-parturient.

The ability to determine the prognosis of a given lesion is extremely important in determining treatment options in both canine and human patients and histopathology is an invaluable tool in this regard. Thus we evaluated eight different morphologic parameters. Individually these parameters were not able to determine either the diagnosis or the prognosis of the lesion, however when viewed in combination a diagnosis could be made. In this study most of the lesions had a ductular growth pattern and were expansile in nature. While only 10 of the 137 lesions contained lymph node for evaluation 6/10 (60%) of those lesions had lymph node involvement. Thus it maybe advisable to recommend lymph node biopsy to further assist in determining the prognosis of a patient. The most common diagnoses were of lobular descent (adenoma, and adenocarcinoma)

and this result is consistent with other reported tumor classifications.[4] It would seem logical that the practice of spaying, and thus removing the hormones responsible for development of this tissue, the risk of cancer may be greatly reduced.

HER-2 is used as a prognostic indicator in human breast cancer where 30% of all tumor types overexpress the protein. In recent studies, HER-2 has been shown to be over expressed in 17% of CMT and 57% of feline tumors, however there has been little to indicate if the over expression of HER-2 can be used as a prognostic indicator in the canine. We therefore examined the expression of HER-2 using immunohistochemistry in an effort to correlate human and canine mammary cancer biology. The results of this study are consistent with others in the literature in that approximately 23% of the lesions in this study highly over expressed the HER-2 protein.[21,22,41] However, our study demonstrated that there is no difference in HER-2 scores and when compared to the aggressiveness of the lesion. One explanation for this result was proposed by Ahern et al (1996); briefly, HER-2 over expression is not needed for canine mammary cell transformation, rather the over expression of HER-2 is a result of the late stage of the disease.[2] Interestingly, Regitnig et al (2004) found that HER-2 status changed with distant metastasis at a later stage of disease.[35] Additionally, HER-2 heterodimers are activators of vascular endothelial growth factor and angiogenesis, characteristic of, and necessary for later disease stage.[55] It is thus conceivable that the randomness of the sample set and the proposed characterization of CMT have led to the result of benign lesions over-expressing HER-2. This may be a key factor in the consideration of using the canine as a model for human breast cancer research.

The median time to reoccurrence was decreased in the HER-2 over-expressing group by 109 days in our study. However, there was no evidence that the lesion at the time of the disease event predicted the morphologic diagnosis of the reoccurring lesion. Moreover, when HER-2 was over expressed, survival time decreased by 276d. Indeed both canine and human studies have shown that patients with lesions over-expressing HER-2 have a shortened disease free interval as well as a shortened survival time indicating that HER-2 may indeed be of prognostic significance in CMT, similar to what occurs in human tissues.

CAV-1 is a major component in the creation of invaginations in the membrane of cells. Additionally this protein acts as a regulator of down stream cell signaling, effectively slowing cell growth. CAV-1 is aberrantly expressed in approximately 16% of human breast cancer lesions. However, little if any research has looked into the protein expression in canine mammary cancer. We have therefore examined the expression of CAV-1 using immunohistochemistry in canine mammary cancer.

CAV-1 has been identified as a tumor suppressor gene in various human cancers. Additionally, CAV-1 is shown to interact with HER-2 blocking signal transduction by the tyrosine kinase.[33] On the other hand Chen et al (2004) found no interaction between HER-2 and CAV-1.[8] In this study we show that 31% of the CMT examined had little or no CAV-1 expression, this is similar to other studies in human tissue.[8,49] These lesions did not differ significantly form those over-expressing CAV-1 in terms of malignancy (Figure 3.10). HER-2 and CAV-1 showed no interaction in this study, which is in agreement with the findings of Chen et al (2004).[8] Other studies have shown that 16% of human breast cancers have a mutation of CAV-1,[14,17] however these studies did not

evaluate the protein expression of CAV-1. CAV-1 is a proposed tumor suppressor gene, its mutation would therefore increase the tumorogenesis of cells were it is aberrantly expressed and may increase the aggressiveness of the lesion. However, in the CMT examined in this study, those that over expressed CAV-1 showed a decrease in median disease free interval of 236 d as well as median survival time of 292 d. One explanation may be that CAV-1 shows differential expression within different stages of breast cancer.[89] Another explanation of this result could be that our recheck time period for most cases was limited to 2.5 years. Additionally, only 39 of the 140 cases reported reoccurrence data while 15 of 140 cases reported survival data. Moreover, different ethnicities of people show differences in pathology[6,85,8] thus it is not out of the realm of possibility that CAV-1 may not show the same pattern of expression in human and canine cancer, or even between different breeds within the canine species.

Finally, ER has been widely used as a prognostic indicator in both human and canine mammary cancer.[16,20,27,4526,30,35,50] However, many of these studies only identify nuclear ER activation. Recently there has been interest in rapid steroid responses, most likely due to steroid receptors that reside in and on the plasma membrane.[32,46,50] The plasma membrane binding of these receptors is thought to activate several cell signaling cascades including those that are associated with G-proteins,[31] SRC and Ras.[23,24] Additionally, ER is closely associated with CAV-1 in the cytoplasm and CAV-1 is thought to be part of the mechanism that translocates ER to the plasma membrane. In the current study, we evaluated both the traditional nuclear expression of ER as well as ER cytoplasmic potential to provide useful prognostic data for CMT. Previous studies have shown benign lesions are more likely to be ER nuclear positive than those that are

malignant .[21,28] In contrast we found no difference in ER positivity in the nucleus between benign and malignant lesions. However, cytoplasmic positivity for ERα has good prognostic value in CMT in our study; those patients with lesions positive for ERα in the cytoplasm were more likely to have benign lesions. Unfortunately there were too few data points to accurately estimate survival time or time to reoccurrence.

Additionally, protein interactions between HER-2, CAV-1 and ERα were evaluated. In human studies it is thought that CAV-1 may help to locate ERα in the region of the plasma membrane.[30] Thus lesions that are positive for ERα in the cytoplasm may also over-express CAV-1, though in this study we found no correlation between ERα cytoplasmic positive lesions and over expression of CAV-1. However, this does not necessarily mean that CAV-1 and ERα do not interact in the cytoplasm of the cell. The fact that CAV-1 is a proposed tumor suppressor and ER positivity in the cytoplasm in this study has a longer disease-free interval, may provide evidence for decreased survival and time to reoccurrence of human patients with aberrant expression of CAV-1. If CAV-1 is unable to mobilize ERα to the plasma membrane, it may negate the positive effects of ERα signaling at the level of the cytoplasm, such as the activation of ERK to create a pro-apoptotic state in breast tumor cells.[5]

There is increasing evidence that HER-2 and ERα interact at the level of the plasma membrane.[46] Briefly, it is postulated that an ERα-estrogen complex is associated with a HER-2/HER-3 heterodimer, causing its activation and down stream effects of the PI3 kinase/ AKT pathway. In the present study there was no association with over expression of HER-2 and cytoplasmic positive ERα lesions, however we did not try to localize such an interaction within our sections.

This study identified if a pattern of protein dysregulation in CMT that could be related to the morphologic characteristics of those lesions. Specifically we looked at the immunohistochemical expression of HER-2, CAV-1 and ERα and demonstrated that none of these proteins were able to predict morphology or benign/malignant status of the lesion save estrogen receptor staining of the cytoplasm. Patients with lesions over-expressing HER-2 and CAV-1 had a shortened disease-free interval and survival times. Patients with lesions staining positive for ERα in the cytoplasm were more likely to have benign lesions. We conclude that HER-2, CAV-1 and ERα are indeed important for determining prognosis of CMT. However the complex interaction of these and other cell signaling mechanisms remains unclear. This study showed no overt interaction between the proteins of interest. This is not to say that these proteins do not impact each other in the development of a neoplastic event. However in the current study we only evaluated immunoreactive protein in the cells of the lesion. We did not measure the functionality of the protein nor did we look at the specific localization of the protein in the cell, specifically bound to the membrane or only sequestered in the cellular compartment. With these unanswered questions we can only speculate as to the interaction of the proteins of interest. Future goals of this research may answer these questions so that we may better understand the actions of HER-2 CAV-1 and ERα in canine mammary tumors. Additional studies to unravel the interactions of these proteins will remain important to better prognostic indices and develop adjunct therapy.

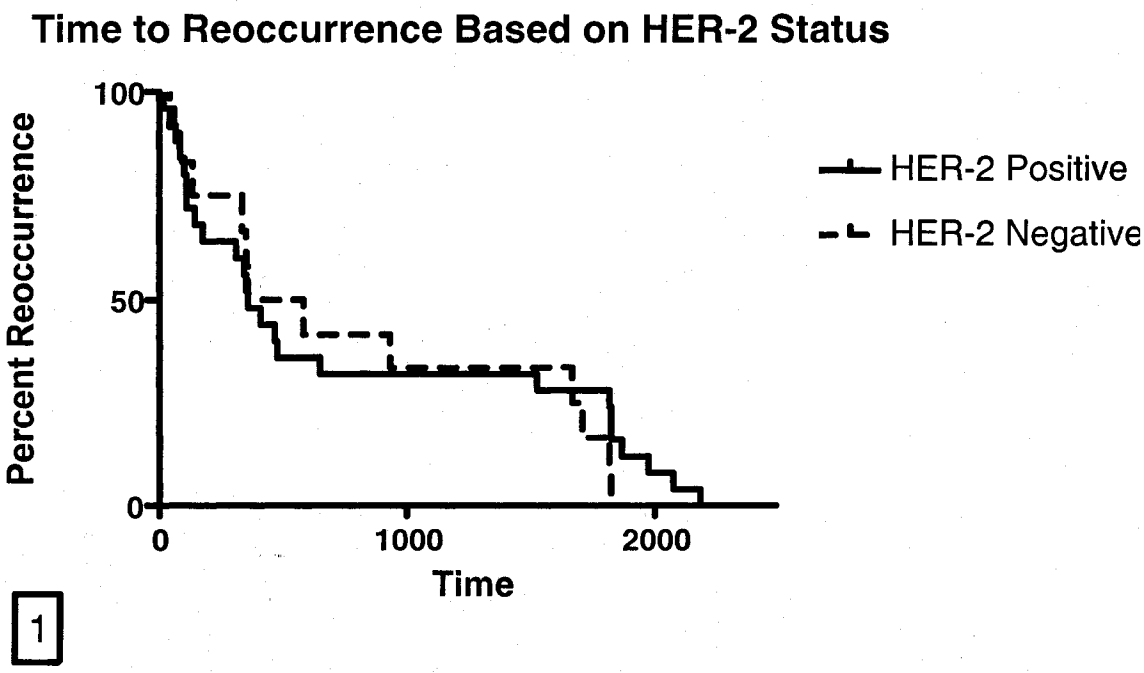


Fig 3.1. Kaplan Myer graph showing the decreased in time to reoccurrence of HER-2 over-expressing lesions. The time interval reported by the referring veterinarians was 23-1709d. The median time to reoccurrence was decreased by 109d in the HER-2 positive group.

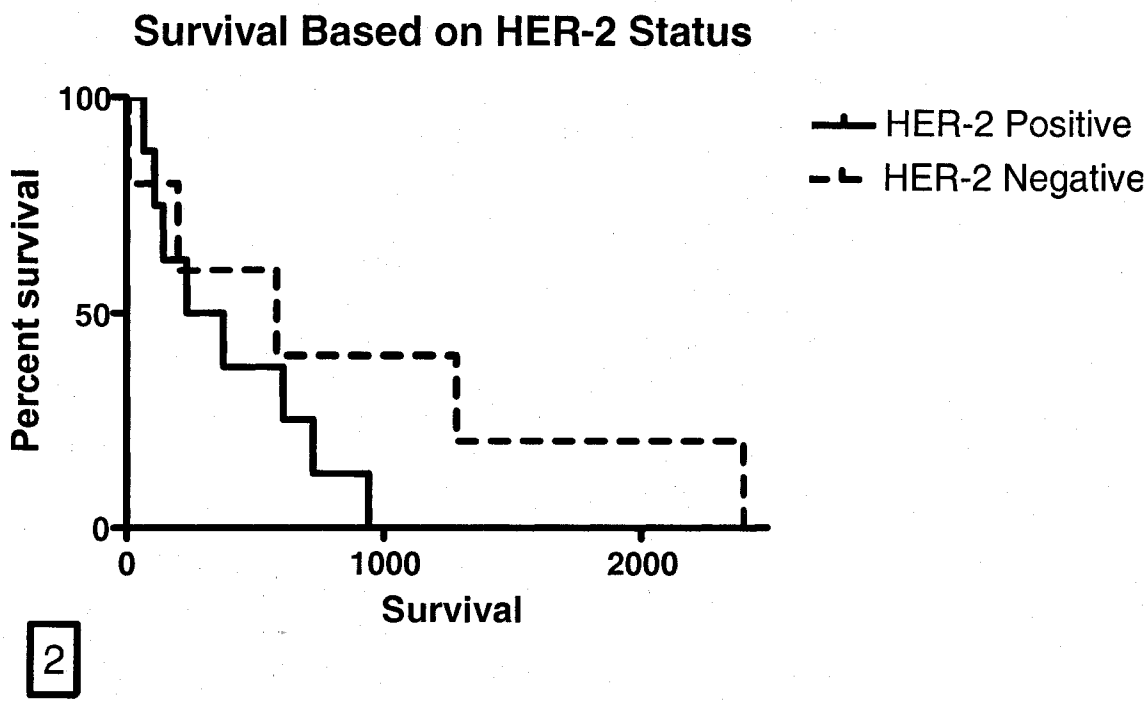


Fig 3.2. Kaplan Myer graph showing the decrease in median survival time of HER-2 over-expressing lesions. The survival time reported by the referring veterinarians ranged for 9-2402d. The median survival time decreased by 276d in the HER-2 positive group.

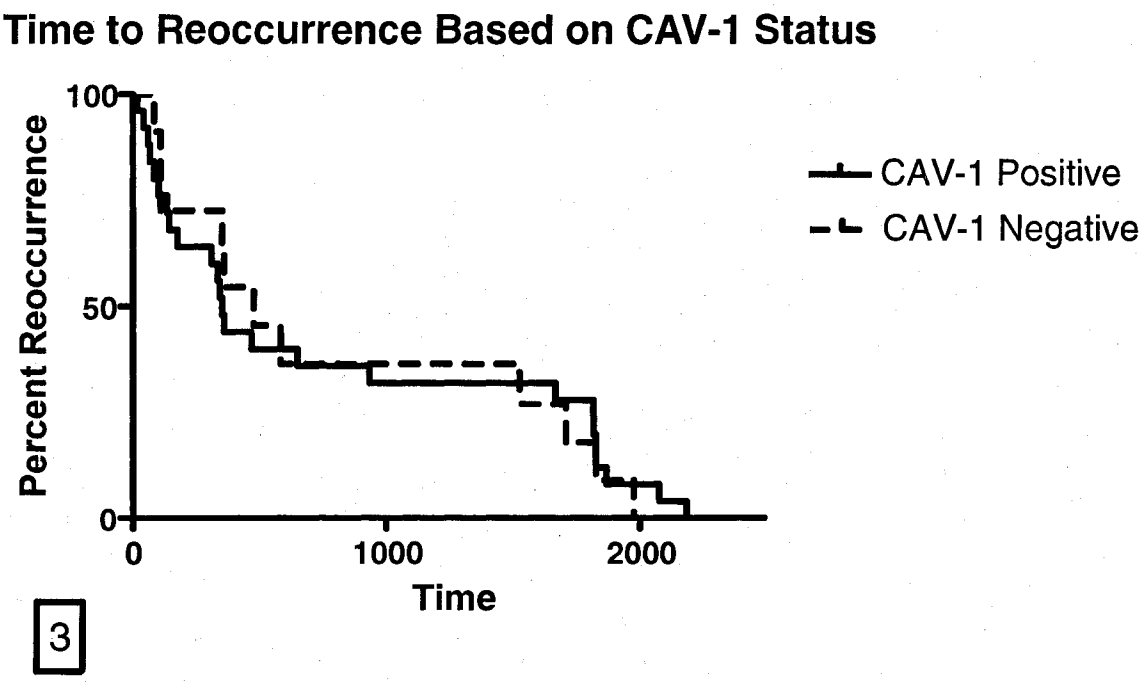


Fig 3.3. Kaplan Myer graph showing the decreased in time to reoccurrence of CAV-1 positive lesions. The time interval reported by the referring veterinarians was 23-1709d. The median time to reoccurrence was decreased by 236d in the CAV-1 over-expressing group.

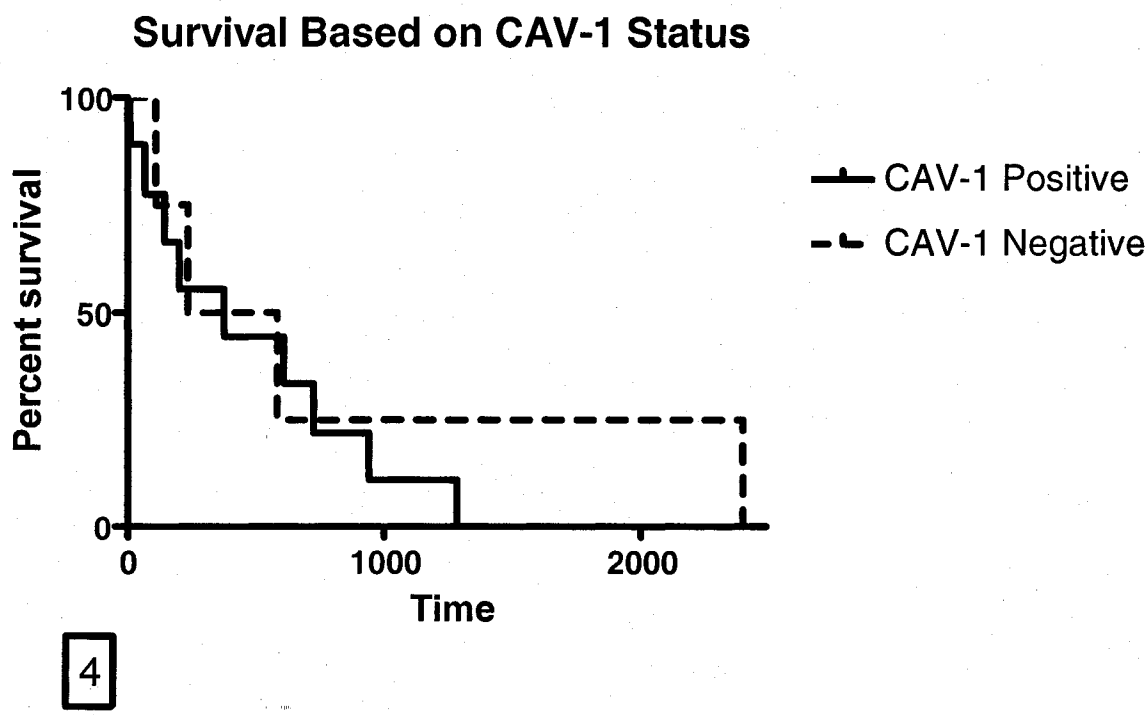
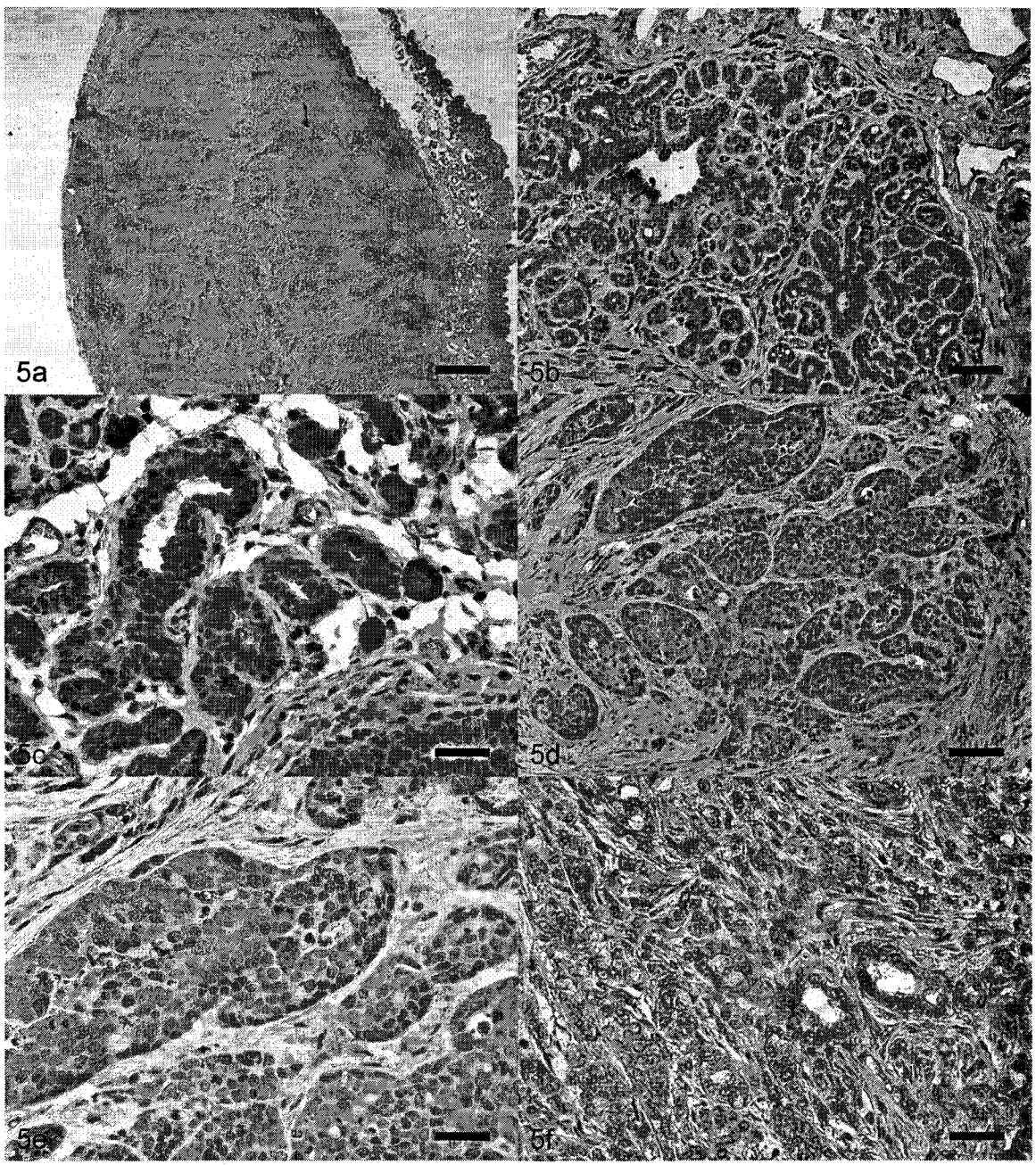


Fig 3.4. Kaplan Myer graph showing the decrease in median survival time of CAV-1 over-expressing lesions. The survival time reported by the referring veterinarians ranged for 9-2402d. The median survival time decreased by 292d in the CAV-1 positive group.



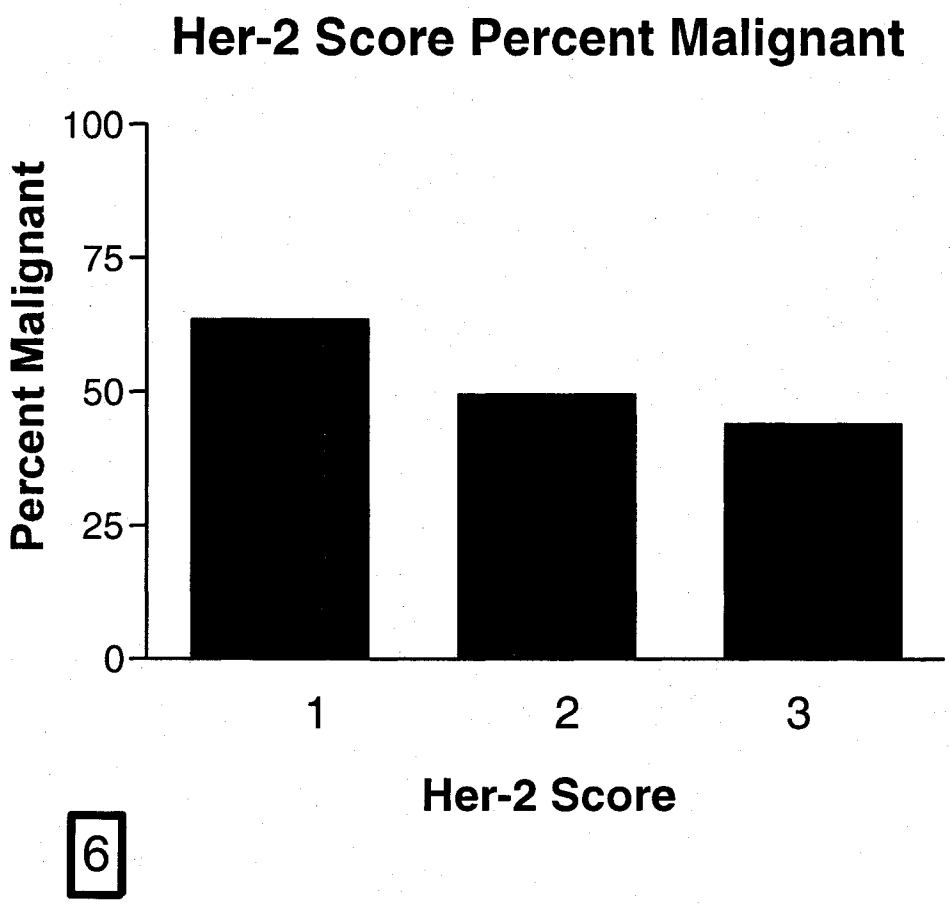


Fig 3.6. Shows the decreasing trend of percentage malignant lesions as HER-2 score increases.

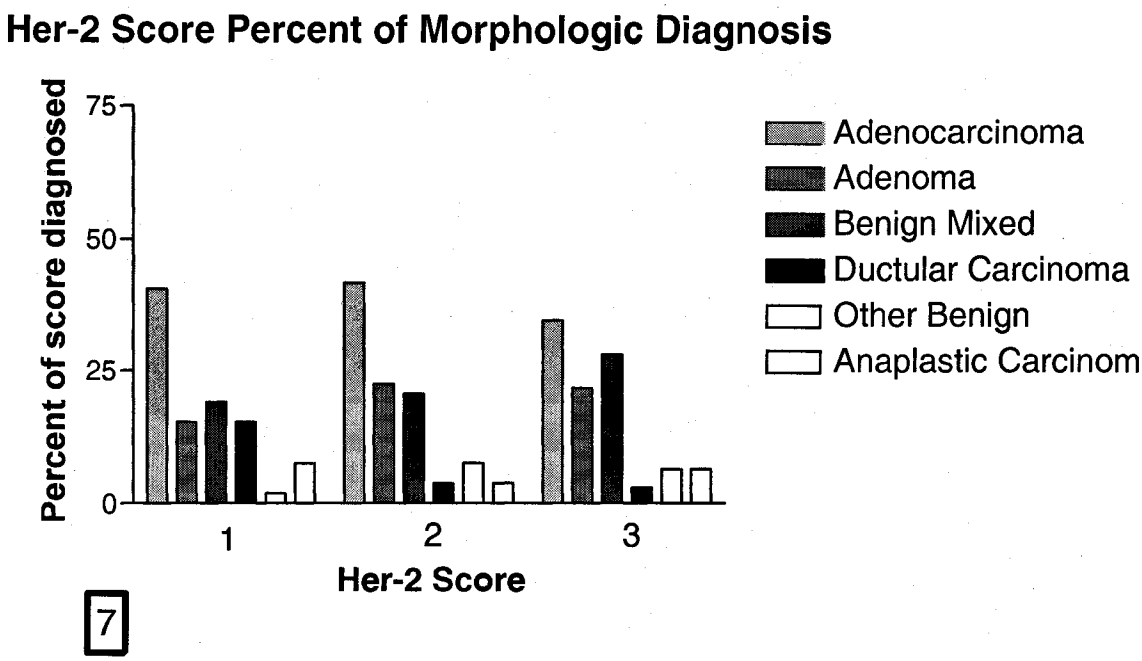
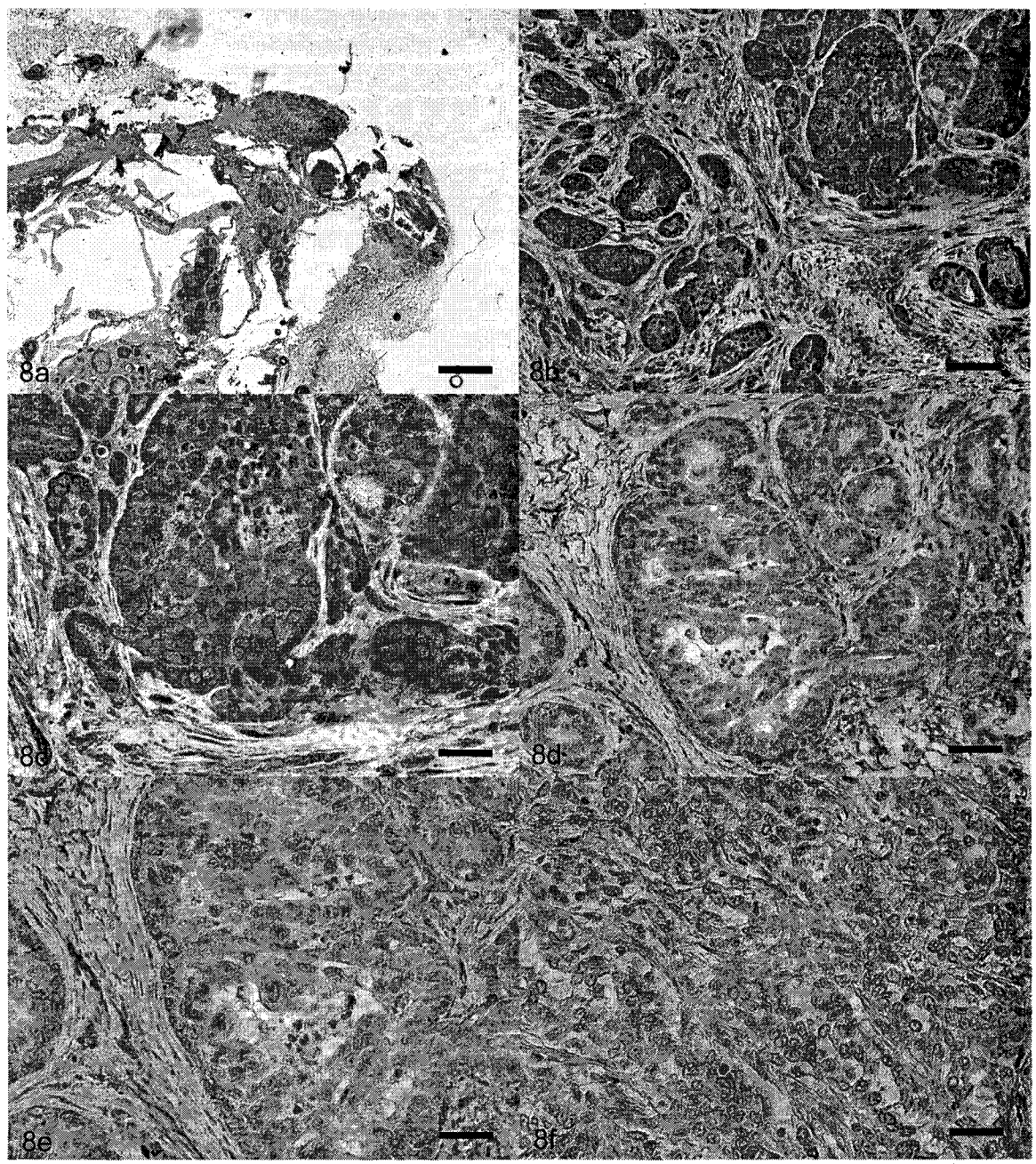


Fig 3.7. The relationship of morphologic diagnosis compared to HER-2 score.



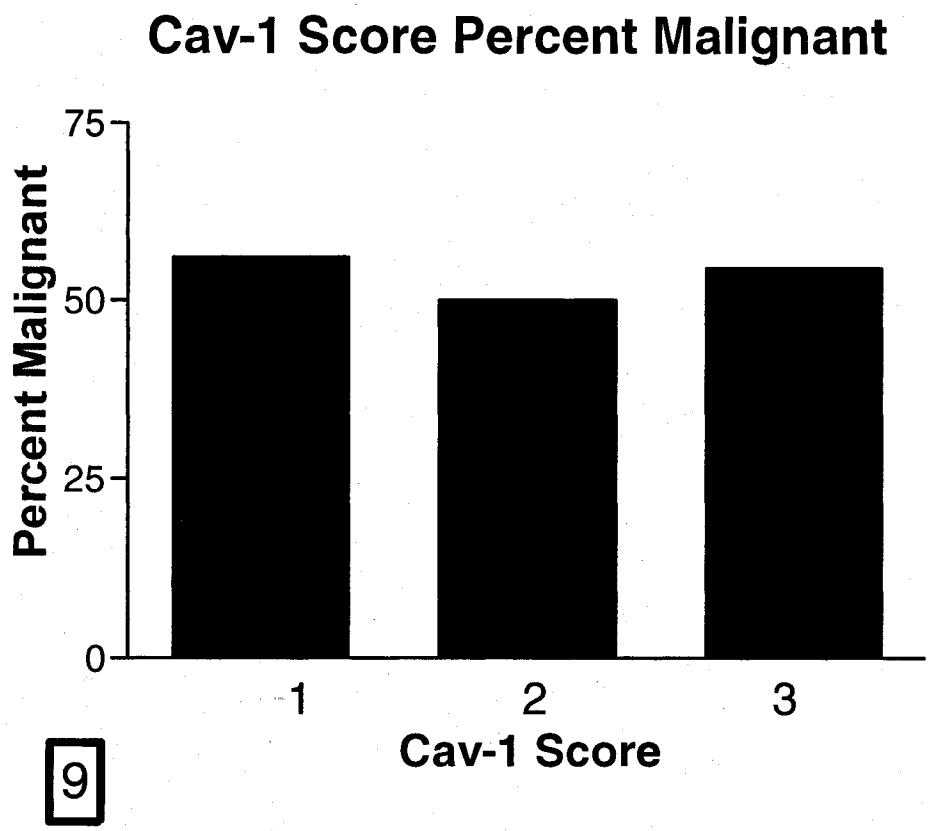


Fig 3.9. Shows the percentage malignant lesions compared to CAV-1 score.

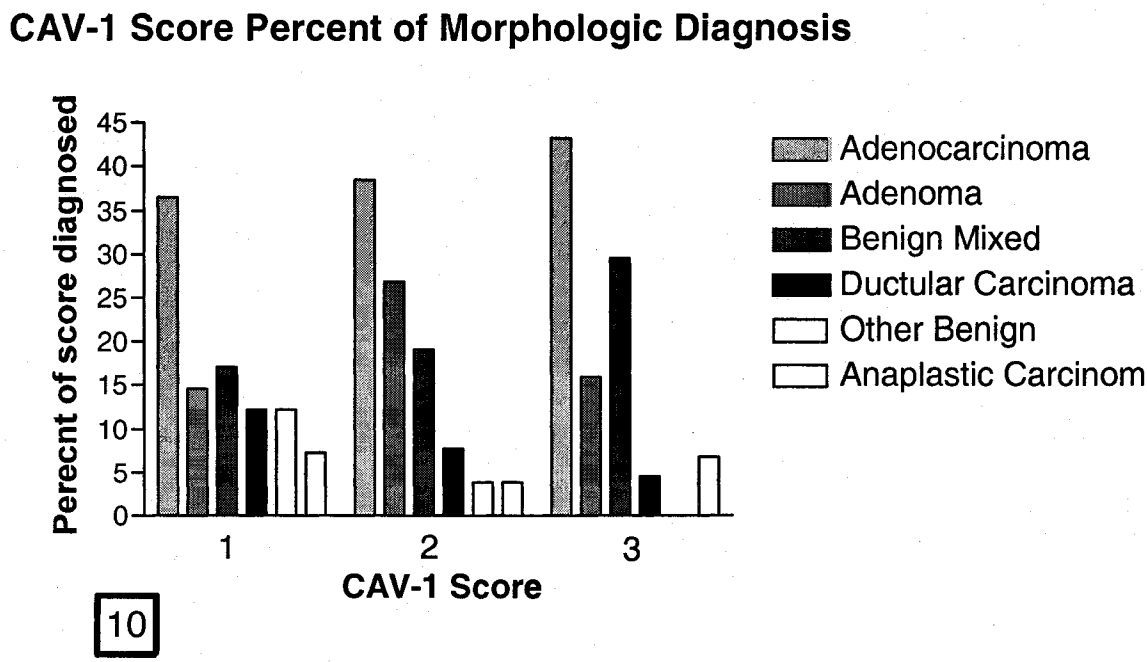


Fig 3.10. Shows the relationship of morphologic diagnosis compared to CAV-1 score.

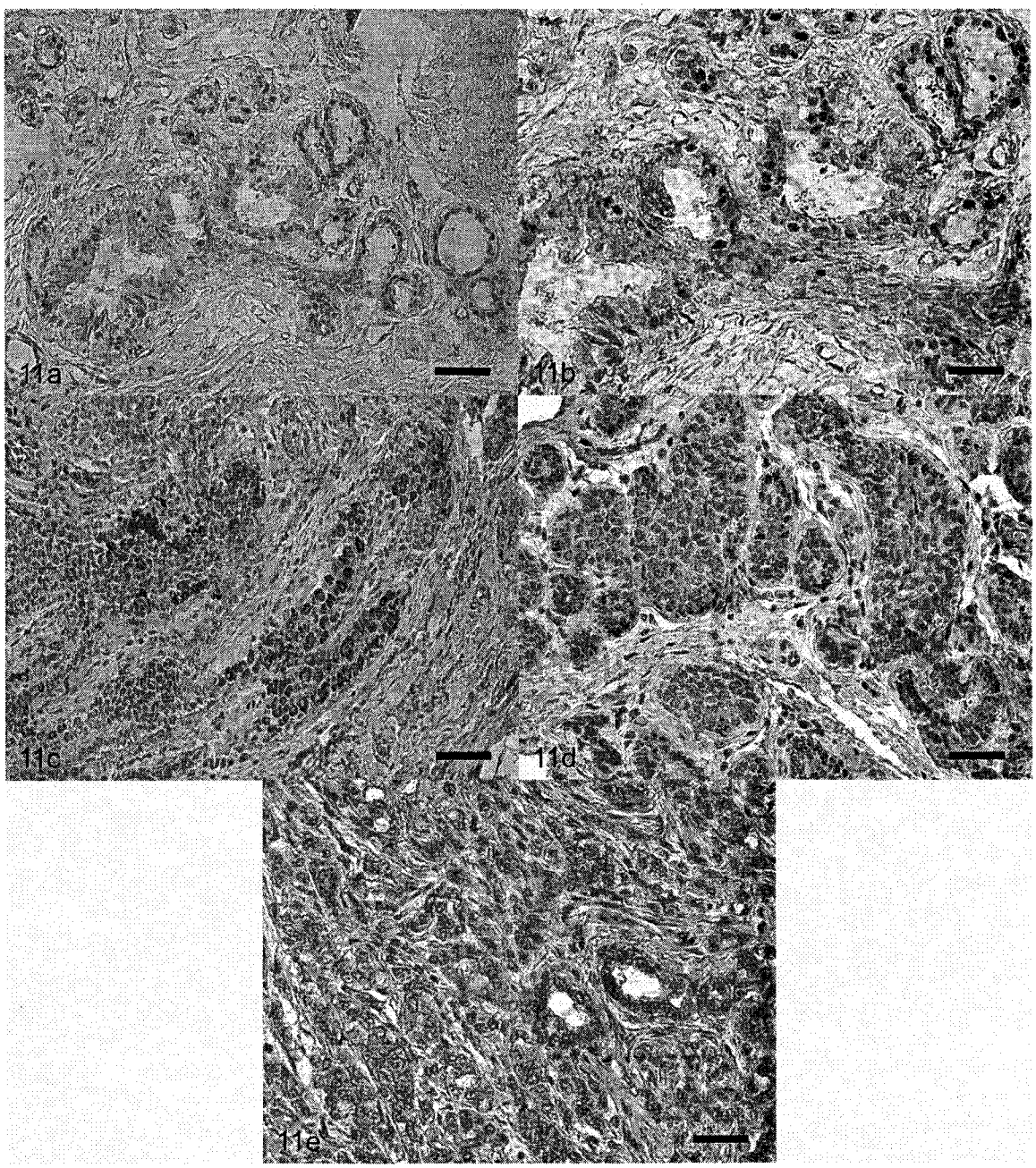


Table 3.1: Breed Representation

Breed	Number	Breed	Number	Breed	Number
AIREDALE	1	FOX TERRIER	1	PUG	1
AKITA	1	GERMAN SHEPARD/ GERMAN SHEPARD X	11	RHODIZIAN RIDGEBACK	1
BEAGLE/BEAGLE X	25	GERMAN WHITE HAIR	1	ROTTWEILER	2
BOUVIER	2	GOLDEN RETRIVER	3	SALUKI	1
BRITTNEY SP.	4	GREAT DANE	1	SCHIPPERKE	1
BULLDOG	1	GREAT PYRNESE	1	SCHNAUZER	2
CHIHUAHUA	4	HUSKY	6	SHIH TZU	3
CHOW X	1	IRISH SETTER	1	SILKY TERRIER	1
COCKAPOO	2	KEESHOND	1	SPANIEL X	1
COCKER SP./COCKER SP. X	8	LABORADOR/ LABORADOR X	12	SPRINGER SP.	3
CORGI	2	LHASA	2	TERRIER/TERRIER X	2
DACHSUND	3	MALTESE	1	WEIMARANER	1
DALMATION	1	MINITURE POODLE	3	WEST HIGHLAND TERRIER	2
ENGLISH SETTER	1	MIXED	3	WIREHAIR VIZSLA	1
ENGLISH SP.	2	POODLE	4	YORKIE	4

Table 3.2: Age Distribution

Age	Number
1-4	4
5-6	8
7	23
8	16
9	13
10	10
11	12
12-13	15
14 <	7

Table 3.3: Mature versus Young Patients and Morphology

	AGE	
	<6 yrs of age	>6 yrs of age
Adenocarcinoma	3	43
Adenoma	2	19
Anaplastic Carcinoma	0	5
Benign Mixed	6	17
Ductular Carcinoma	0	8
Other Benign	1	3

Table 3.4: Body Condition Score

BCS	Number
1	0
2	1
3	29
4	23
5	4

Scored for 1-5 (1- emaciated 5-obese). Numbers of refering veterinarian survay.

Table 3.5: Body Condition Score Across Tumor Types

	Body Condition Score			
	2	3	4	5
Adenocarcinoma	1	14	8	0
Adenoma	0	4	4	1
Anaplastic Carcinoma	0	0	1	0
Benign Mixed	0	9	7	1
Ductular Carcinoma	0	1	2	2
Other Benign	0	1	1	0

Table 3.6: Sex Distribution

Sex	Number
Female	79
Spayed Female	58
Neutered Male	2

Table 3.7: Morphologic Diagnosis of Mammary Sections

Diagnosis	Number
Adenocarcinoma Complex	14
Adenocarcinoma Simple	40
Adenoma Simple	6
Adenoma Complex	21
Bengin Mixed	32
Ductular Carcinoma	4
Ductular Hyperplasia	2
Ductular Papiloma	4
Ductular Papilary Carcinoma	7
Lobular Hyperplasia	2
Anaplasic Carcinoma	8

For statistical analysis the morphologic diagnosis of the following were combined: Simple and Complex Adenocarcinoma as adenocarcinoma; Simple and Complex Adenoma as Adenoma; Lobular hyperplasia, Ductular hyperplasia and Ductular papiloma as Other bengin; Ductular carcinoma and Ductular papilary carcinoma as Ductular carcinoma. These diagnosies were combined because of there simliar biologic nature

Table 3.8: Sex and Morphologic Diagnosis

	SEX	
	Intact	Altered
Adenocarcinoma	23	30
Adenoma	21	6
Anaplastic Carcinoma	7	1
Benign Mixed	15	14
Ductular Carcinoma	6	5
Other Benign	5	2

Table 3.9: Growth Pattern Observed in Mammary Sections

Growth Pattern	Number of cases
Ductular	51
Ductular/Papillary	15
Ductular/Solid	24
Ductular/Papillary/ Solid	1
Papillary	14
Papillary/Ductular	2
Papillary/Ductular/Solid	9
Solid	10
Solid/Ductular	9
Solid/Papillary	2
Solid/Ductular/Papillary	1

The first growth pattern predominated in the section, the other growth patterns were also characteristics of the section.

Table 3.10: Invasion Observed in Mammary Sections

Invasion	Number of Cases
Expansile	67
Local	33
Regional	1
Vascular	2
Nodal	1
Regional w/ vascular component	18
Vascular w/ nodal component	2
Regional w/ vascular & nodal component	1

The first invasion pattern predominated in the section, the other invasion patterns were also characteristics of the section.

Table 11: Morphologic Diagnosis of Primary and Reocurring Lesion

Morpholoic Diagnosis of Primary Lesion	Histopathology of Reocurring Lesion
Adenocarcinoma	Adenocarcinoma
Adenocarcinoma	Adenocarcinoma
Ductular Papiloma	Adenoma
Bengin Mixed	Adenoma/Adenocarcinoma
Adenocarcinoma	Adenocarcinoma
Adenocarcinoma	Ductular Carcinoma

Table 3.12: Time From Spay to Event

Time (mo)	Number
<12	29
13-24	4
25-36	3
37-60	8
>61	4

Number from refering veterinarian survay

Table 3.13: Time to neuter and Morphologic Diagnosis

	Time to Neuter <12mo
Adenocarcinoma	10
Adenoma	6
Anaplastic Carcinoma	1
Benign Mixed	11
Ductular Carcinoma	0
Other Benign	1

Table 3.14: Breeding Female and Morphologic Diagnosis

	Breeding Female	
	Yes	No
Adenocarcinoma	11	22
Adenoma	5	17
Anaplastic Carcinoma	0	1
Benign Mixed	5	14
Ductular Carcinoma	2	6
Other Benign	0	6

Table 3.15: HER-2 Score Distribution

HER-2 Score	Number
1	52
2	53
3	32

Table 3.16: Bengin/malignant based on Protein Status

	ER α Cytoplasmic Status		ER α Nuclear Status		HER-2 Score			CAV-1 Score		
	Positive	Negative	Positive	Negative	1	2	3	1	2	3
Bengin	20	29	11	38	19	27	18	18	26	20
Malignant	8	42	11	39	33	26	14	23	26	24

Table 3.17: Protein Status and Morphologic Diagnosis

	ER α Cytoplasmic Status		ER α Nuclear Status		HER-2 Score			CAV-1 Score		
	Positive	Negative	Positive	Negative	1	2	3	1	2	3
Adenocarcinoma	5	7	2	14	21	22	11	15	20	19
Adenoma	9	36	10	31	8	12	7	6	14	7
Anaplastic Carcinoma	0	2	0	2	4	2	2	3	2	3
Benign Mixed	9	18	8	19	10	11	9	7	12	13
Ductular Carcinoma	3	4	1	6	8	2	1	5	4	2
Other Benign	2	3	1	5	2	4	2	5	2	0

Table 3.18: CAV-1 Score Distribution

CAV-1 Score	Number
1	41
2	52
3	44

Table 3.19: ER alpha Distribution

ER alpha	Cytoplasmic	Nuclear
Positive	28	22
Negative	71	77

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Summary

Indeed HER-2 is a major player in cell transformation to the neoplastic state. Our as well as many other studies have shown that HER-2 is over expressed in canine mammary tumors and osteosarcoma and well as human beast cancer and osteosarcoma. It is so well documented in fact that there is evidence to support a molecular target adjunct therapy for human breast cancer. This therapy when used in human breast cancers as well as some osteosarcomas has great benefit to the patient. However, these lesions soon become resistant to anti-HER-2 treatment and adopt other cellular mechanisms to continue there neoplastic activity. Lending further evidence that there is no one single treatment for curing a neoplastic lesion. Thus identifying cell signaling interactions unique to neoplastic lesions continues to be the future of cancer treatment research. In the current series of studies we set out to identify protein interactions specific to neoplastic lesions. We used the dog as our model because of their unique similarity to human cancer not only in histomorphology but also in lesion behavior and tumor predilection. We were specifically interested in the interactions of HER-2, CAV-1 and ER α in predicting histomorphology and outcome of the canine patient. Additionally, this research may provide information supporting the use of the canine as a model for human cancers.

Osteosarcoma is one of the most aggressive cancers in canine medicine, having a metastatic rate of 80% on presentation. The current method of treatment relies on resection of the lesion and subsequent radiation/chemotherapy. HER-2 is over expressed in osteosarcoma and is thought to be poor prognostic indictor in human patients. Though osteosarcoma is a non-epidermal origin neoplasia the same mechanisms of down stream cell signaling for growth and development are thought to play a role in the development

of an osteosarcoma lesion.

The current study indicates that canine osteosarcoma indeed over expressed HER-2 both at the protein level indicated by immunohistochemistry and at the molecular level indicated by real time PCR. Additionally, the patients who had lesions over expressing HER-2 had a decrease in median survival of 115d compared to patients with HER-2 negative lesions. This study supports the fact that HER-2 maybe an important indicator of prognosis for canine patient with osteosarcoma. Moreover, canine patient may benefit from the use of anti-HER-2 adjunct therapy to prolong the survival of patients with HER-2 positive lesions. Additionally, the data lends support to the use of companion animals specifically dogs to further our understanding of osteosarcoma in human patients.

Though the data show a positive correlation between HER-2 and prognosis in canine patients the sample size is limited. In order to further validate what we have introduced as a potential model and therapy we must continue to investigate this relationship. In order to best achieve this one should proceed with a prospective study, this would give us the best potential to gather subject data and allow us the ability to better follow up with patients enrolled in the study in order to best evaluate prognosis of patients with HER-2 positive lesions. Secondly, as HER-2 is over expressed osteosarcoma it is not an unsupported assumption to believe that anti-HER-2 therapy would benefit these patients. Thus it would be warranted to identify a study group of canine osteosarcoma patients to receive anti-HER-2 therapy, and compare those patients outcome to a group who did not receive anti-HER-2 therapy. The above considerations along with the current study as well as studies in human medicine regarding HER-2 and osteosarcoma may identify a better multimodal treatment plan directed at molecular

targets to control this aggressive cancer.

Breast cancer is one of the most common cancers diagnosed in both human and veterinary medicine. The current therapy in both human and canine medicine remains surgical followed by radiation/chemotherapy. However, in human medicine molecular adjunct therapy has achieved a toehold in multimodal treatment plans. Still we rely on treating a molecular disease with a scalpel blade. Approximately 25-30% of all human breast cancers and canine mammary cancers over- express HER-2. Several current studies have begun to look into the complex interactions of HER-2 and other cell signaling mechanisms including those of CAV-1 and ER α and have found evidence to support these interactions. The down stream signaling of HER-2 though the AKT/PKB and pathway stimulates cell proliferation, limits apoptosis, stimulates angiogenesis and leads to the development of a neoplastic lesion.

The current study looks into the interactions of HER-2, CAV-1 and ER α in canine mammary tumors. HER-2 was over expressed in 23% of the neoplasias in our study. Moreover, patients with HER-2 positive lesions had a poor prognosis having a 109d decrease in time to reoccurrence and a 276d decrease in survival time. CAV-1 was over expressed in 32% of the lesions in our study, these patients had a poor prognosis with a decrease in median time to reoccurrence of 236d and a decrease in median survival time of 292d. This differs from the human literature where aberrant methalyation of CAV-1 creates a decrease in the active form of CAV-1 and causes sequestration in the cytoplasm. This may in fact be the case in these lesions, as we only identified the amount of CAV-1 in the lesion. It could be that the lesions over expressing CAV-1 are those that do not have functional CAV-1. Finally, 22% of the lesions were positive for ER α in the

nuclear compartment. Interestingly, 28% of the lesions were positive for ER α in the cytoplasm. Of these lesions 71% were benign in nature while only 40% of the lesions ER α negative in the cytoplasm were benign. Taken together we had identified several possibilities of prognostic indicators and possible therapeutic targets of canine mammary tumors. Additionally, our study provides increased support for using the canine as a model of human breast cancer.

While the goal of this study was to identify protein interactions between HER-2, CAV-1 and ER α, no correlation was found between the relative expression of one protein compared to another. This is not to say that these proteins do not interact. It is to say there is no direct cause and effect relationship between them. With that said further work in this area should include gene expression studies of these proteins. It maybe that over expression of HER-2 acts not on the expression of ER α itself but rather estrogen response elements, or possibly causes the aberrant methylation of CAV-1. These are questions that must be answered not only to better understand how these proteins interact in the cellular environment to produce a neoplasic lesion, but how we can exploit them to target therapy to better treat an individuals cancer.

There have been many huge steps in the development of caner research and treatment. This has lead to more directed therapy and longer survival times in patients afflicted with the disease. Still we are in the infancy of our molecular understanding of cancer biology. As we proceed in identifying the underlying molecular changes needed to develop a neoplasic lesion we must remember that a complex system requires a complex answer. Targeting only a specific aspect of a lesion will only result in resistance

to the treatment. Thus we need to develop multimodal therapies that target and individuals cancer specific molecular make up. Only then can we cure cancer.