RELATIVE HER2 PROTEIN EXPRESSION IN NORMAL CANINE TISSUE AND CANINE OSTEOSARCOMA

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

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RELATIVE HER2 PROTEIN EXPRESSION IN NORMAL

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ABSTRACT

Development of HER2-targeted immunotherapy in canine osteosarcoma (OSA) is underway, yet HER2 expression in normal and neoplastic canine tissue has not yet been defined. This study's objective was to evaluate the relative HER2 protein expression in canine osteosarcoma tissue, normal canine tissue, and a HER2-overexpressing human cancer cell line.

Flash frozen tumor tissue donated from 24 dogs with spontaneously-occurring OSA tumors, 2 normal colon samples, cell pellets from 2 OSA cell lines (Abrams, HMPOS) and a HER2-overexpressing human cell line were available for protein extraction and HER2 immunoblotting. Formalin-fixed tissue suitable for H&E staining and HER2 immunostaining was available for 6 normal colon and 18 OSA (13 primary tumors, 5 metastases) canine samples. None of the 26 OSA tumors showed relative HER2 blot band intensity equal to or greater than those of controls. Four of 5 OSA metastases and all 13 OSA primary tumors showed some degree of HER2 immunostaining, predominantly weak and cytoplasmic. Only a subset of spontaneously occurring canine osteosarcoma tumors express HER2 in a relevant manner for treatment with anti-HER2 immunotherapy, and not in a membranous pattern to justify surface-targeted therapies.

INTRODUCTION

EGFR Family

As a member of the epidermal growth factor receptor family, HER2 (human epidermal growth factor receptor-2, also neu, erbB2) contains an extracellular component with regions for dimerization and ligand binding, a short transmembrane domain, and an intracellular domain with tyrosine kinase capability. While erbB1 (EGFR) and erbB4 (HER4) are able to homodimerize and successfully initiate signaling cascades, erbB2 (HER2) and erbB3 (HER3) are obligated to heterodimerize with other growth factor receptors. HER2 has no known high affinity ligand, and erbB3 lacks the intracellular tyrosine kinase domain.(Harari & Yarden, 2000)

The EGF family receptors convert input in the form of ligands to signaling through tyrosine kinase activity, with the final output of altered gene expression. The final signaling outcome depends heavily on the specific ligand, dimerization pattern, and other environmental signaling. Ligands include TGF-alpha, EGF, epiregulin, beta-cellulin, HB-HGF, amphiregulin, and NRG 1-4. The most studied mitogenic pathways activated are RAS/MAPK and PI3K/AKT, resulting in signaling for proliferation, angiogenesis, and migration.(Yarden & Sliwkowski, 2001)

While HER2 does not avidly bind growth factors directly, it enhances ligand binding with EGFR, HER3, and HER4 when in the heterodimer state. Overexpression of HER2 further increases ligand binding affinities to the other receptors. In addition to enhancing ligand affinity, HER2 overexpression slows

the rate of ligand dissociation from EGFR family heterodimers.(Karunagaran et al., 1996) The net effect of prolonged ligand-receptor complex life span is increased activity of intracellular signaling cascades.(Graus-Porta, Beerli, & Hynes, 1995) Ligand-receptor binding, receptor dimerization, and therefore downstream signaling cascade effects, are not random; they are controlled by tissue-specific production of ligands and receptor-type expression. In fact, HER2 has superior ability to form heterodimers compared to the other receptors in the EGFR family.(Tzahar et al., 1996)

In addition to the classic heterodimerization of HER2 with the other epidermal growth factor receptors, HER2 can interact with other receptor families, including MUC1 and Ob-R (leptin receptor). Interaction with MUC1 results in activation of the PI3K/AKT pathway, and triggers MUC1 to translocate to the nucleus where it acts as a transcription factor for the Wnt oncogenes.(Kufe, 2013) Ob-R and HER2 are co-expressed in breast cancer, and HER2 can be transactivated and phosphorylated in the presence of leptin. This relationship has been implicated as a component of increased breast cancer risk in obese women.(Fiorio et al., 2008)

The prototypical function of growth factor receptors as exclusively plasma membrane proteins has been challenged in the last decade. Intact and fragmented growth factor receptors are transported to the nucleus through a variety of mechanisms.(Mills, 2012) The HER2 receptor contains a nuclear localization sequence, and can undergo clathrin-dependent and -independent endocytosis with early endosome trafficking to the nucleus through complex

formation with importin and nucleoporins.(Giri et al., 2005) A HER2 associated sequence (HAS) was identified upstream of the COX-2 gene promoter, where HER2 association up-regulates COX-2 expression.(Wang et al., 2004)

Species differences

While human and rodent erbB2/HER2/neu share 88% sequence homology, subtle yet significant differences exist between the structure and function of the orthologs. The neu receptor tyrosine kinase and proto-oncogene, later found to be synonymous with erbB2 and human epidermal growth factor receptor (HER2), was first described in rats in the early 1980s when an oncogenic variant was discovered in chemically-induced neuro- and glioblastoma.(Bargmann, Hung, & Weinberg, 1986b; Schechter et al., 1984) Malignant transformation was associated with a gain-of-function mutation (c.2014T>A, V664E) in the transmembrane domain of the c-neu protein product. This SNV induces a change in receptor conformation that mimics ligand binding, enhancing receptor dimerization and tyrosine kinase activity in the absence of appropriate signals.(Bargmann, Hung, & Weinberg, 1986a; Weiner, Liu, Cohen, Williams, & Greene, 1989) While essential in oncogenic activation of neu, constitutive activation alone is not sufficient for transformation for the rat ortholog. In transgenic mouse models with mammary expression of rodent neu (wildtype) and activated rodent neu (V664E), both gene amplification (increased expression) and mutation (enhanced signaling) are necessary for tumorigenesis.(Andrechek et al., 2000; Guy et al., 1992; Siegel & Muller, 1996)

In the same time frame, two separate laboratories identified over-expression of an EGFR-like protein, c-erBb-2 (or human epidermal growth factor receptor 2/HER2) in human mammary carcinoma, salivary adenocarcinoma, and a gastric cancer cell line.(King, Kraus, & Aaronson, 1985; Yamamoto et al., 1986) HER2/c-erbB-2/neu were soon identified as orthologs in rat and human species, with noteworthy differences in oncogenic activation.

Mutational activation of membranous or juxtamembranous domains of neu (such as V664E) is required for malignant transformation in the rodent, but not human, ortholog.(Hung, Schechter, Chevray, Stern, & Weinberg, 1986) A 5-10 fold increase in expression of wildtype human HER2 ortholog alone, as is typically seen with gene amplification in HER2 positive breast cancer, is adequate for high efficiency transformation.(Di Fiore et al., 1987)

The necessity of the mutational activation in rats may result from differences in coding sequences between orthologs. The human ortholog of the rodent neu variant c.2014T>A results in V664A rather than V664E. In comparison, the canine HER2 coding sequence at this locus is homologous to humans (GTG in rats, GTT in humans and dogs). While experimentally generated human HER2 V664A and V664E variants demonstrate increased receptor dimerization and activation, these variants are not typically found in spontaneously occurring human tumors.(Di Fiore et al., 1987; Moasser, 2007)

A proposed explanation for the oncogenic potential of human HER2 without mutational activation is the existence of alternative splice isoforms with unique functions. Human HER2 has known splice variants that lead to alternative

translation initiation, post-transcriptional modifications that have been shown to lead to truncation or cleavage of the extracellular domain of the HER2 receptor, as well as a product that lacks exon 16 (Δ16HER2).(Dittrich, Gautrey, Browell, & Tyson-Capper, 2014) Exon 16 overlaps the juxtamembranous domain of the protein that typically requires mutational activation in rodent neu.(Mitra et al., 2009) Δ16HER2 and can form stable constitutively active homodimers in the absence of bound ligand, and contains the epitope recognized by trastuzumab, a therapeutic monoclonal antibody for HER2-positive breast cancer. These splice variants evade trastuzumab binding while maintaining the ability to dimerize and initiate intracellular signaling.(Dittrich et al., 2014; Vu & Claret, 2012)

HER2 as a therapeutic target in human cancers

The structure, function, and possible therapeutic targets of HER2 have been extensively researched over the last three decades due to its overexpression in human breast, esophageal, gastric, ovarian, endometrial, and other cancers.(Iqbal & Iqbal, 2014) HER2 is overexpressed in 25-30% of breast cancer, where it is associated with a more aggressive biologic behavior.(Slamon et al., 1987; Slamon et al., 1989) The best established mechanism of HER2 overexpression in human breast cancer is gene amplification, with neoplastic cell populations containing 25 to even 100 gene copies identified with fluorescence *in situ* hybridization (FISH),(Kallioniemi et al., 1992) however, other mechanisms for overexpression exist.

In addition to FISH, HER2 overexpression can be assessed through immunohistochemistry, however, positivity falls into spectra of staining intensity

and patterns.(Owens, Horten, & Da Silva, 2004; Pauletti et al., 2000) This disparity has necessitated establishment of the 2007 guidelines for HER2 status testing, which combine both IHC and FISH to improve sensitivity, specificity, and reproducibility in equivocal cases.("Guideline Summary: American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor HER2 Testing in Breast Cancer," 2007) With the development of trastuzumab (Herceptin), an FDA-approved anti-HER2 monoclonal antibody for advanced metastatic HER2-positive breast cancer, accurate identification has become essential in decision-making for both cost-effective and medically indicated treatment.(Eiermann & International Herceptin Study, 2001) Trastuzumab binds to the extracellular domain of HER2 and prevents dimerization, suppressing activation of the AKT/MAPK pathways. The antibody can also lead to HER2 ubiquitination, internalization, and degradation. Trastuzumab coating of HER2 over-expressing cells activates the innate immune system, triggering destruction by natural killer cells (antibody-dependent cellular cytotoxicity, ADCC).(Vu & Claret, 2012)

Trastuzumab and newer generation HER2-targeted therapies (ie, pertuzumab, lapatinib) have been associated with cardiac dysfunction.(Crone et al., 2002; Odiete, Hill, & Sawyer, 2012; Schneider, Chang, & Rocco, 2001) The cardiotoxicity of these drugs is attributed to on-target off-tumor effects given HER2's indispensable role in cardiac development, signaling, and repair. HER2 knockout or conditional mutation models in cell lines, mice, rats, and dogs have shown developmental abnormalities in the nervous and cardiac systems,

including fatal failure to form cardiac trabeculae, failure of atrial function and signal propagation, improper patterning of coronary veins, glial cell and ganglia hypoplasia, and poor endothelial development.(Kuramochi, Guo, & Sawyer, 2006; Lee et al., 1995; Lemmens, Segers, Demolder, & De Keulenaer, 2006; Ozcelik et al., 2002; Tenin et al., 2014; Wadugu & Kuhn, 2012) HER2 blockade is associated with diminished myocardial repair, cardiomyocyte death, and the development of dilated cardiomyopathy.(Gordon et al., 2009; Liu et al., 2006) HER2 activation in ischemic, dilated and viral animal models of cardiomyopathy is associated with survival benefits and improvement in cardiac function.(Doggen et al., 2009)

In the pivotal 2001 phase III clinical trial demonstrating survival benefit in women with metastatic breast cancer receiving trastuzumab and an anthracycline versus an anthracycline alone, 27% of women receiving combination therapy developed cardiac dysfunction (compared with 8% in the anthracycline alone group).(Cameron et al., 2017) The risk of cardiotoxicity is greatly reduced by administering trastuzumab after completing all chemotherapy and radiation therapy and required a post-chemotherapy LVEF (left ventricular ejection fraction) of at least 55% before enrollment.(Slamon et al., 2001)

Listeria-based anti-tumor vaccines have been developed for the treatment of human papillomavirus associated tumors of the cervix, vagina/vulva, anus, penis, oral cavity and larynx, and oropharynx. Phase I-III clinical trials employing these vaccines are currently underway, with promising early reports.(Miles, Safran, & Monk, 2017) Given the demonstrated overall safety of Listeria vector

immunotherapies and the efficacy of trastuzumab in HER2-positive breast cancer, a Listeria-based HER2 vaccine was developed. The vaccine consists of a highly attenuated strain of Listeria monocytogenes transformed with a chimeric humanized HER2/neu construct containing two extracellular HER2 domains to include most of the known MHC1 epitopes of the human protein. Mice that overexpress HER2/neu and subsequently develop spontaneous mammary tumors were administered the vaccine prior to tumor development. The study found the vaccine was immunogenic, broke tolerance of HER2/neu antigen, and significantly prolonged the time to tumor development.(Shahabi, Seavey, Maciag, Rivera, & Wallecha, 2011)

HER2 expression has been evaluated in pediatric osteosarcoma, and the prevalence of overexpression as well as its utility as a prognostic indicator remains controversial. A portion of the literature reports a high percentage of osteosarcoma are HER2-positive on IHC (up to 80%), where it is associated with a poorer prognosis.(Mardanpour, Rahbar, & Mardanpour, 2016; Onda et al., 1996; Zhou et al., 2003) Alternatively, a growing number of publications has shown a lower prevalence of IHC HER2-positivity in osteosarcoma of 12-44%, with nearly all positive samples showing weak cytoplasmic and nuclear staining in the absence of the classic strong membranous staining seen in breast cancer.(Anninga et al., 2004; Somers, Ho, Zielenska, Squire, & Thorner, 2005; Thomas, Giordano, Sanders, Biermann, & Baker, 2002) Further, in several studies gene amplification has not been identified with FISH/CISH(Anninga et al.,

2004; Somers et al., 2005; Tsai et al., 2004) and PCR assays have amplified no or background amounts of mRNA.(Anninga et al., 2004; Thomas et al., 2002)

A 2012 phase 2 clinical trial evaluating the safety and efficacy of trastuzumab and cytotoxic chemotherapy in pediatric patients with osteosarcoma showed no improvement in progression free interval or overall survival between a HER2 positive group receiving combination immunotherapy and cytotoxic chemotherapy, and a control HER2 negative group receiving cytotoxic chemotherapy alone.(Ebb et al., 2012) The investigators noted that the patients were deemed HER2-positive for membranous, cytoplasmic, and nuclear staining patterns, unlike in breast cancer where only membranous staining is considered.

HER2 as a therapeutic target in canine cancers

Interest in HER2 status of human tumors has resulted in the application of HER2 testing to canine tumors, primarily with initial focus on cancers and therapeutics with translational potential. Numerous studies in both benign and malignant canine mammary tumors and mammary cancer cell lines have yielded mixed results regarding HER2 expression and association with a more aggressive phenotype.(Ahern, Bird, Bird, & Wolfe, 1996; Hsu, Huang, Liao, Wong, & Chang, 2009; Martín de las Mulas, Ordás, Millán, Fernández-Soria, & Ramón y Cajal, 2003; Murua Escobar, Becker, Bullerdiek, & Nolte, 2001; Rungsipipat et al., 1999) The methodology used to assess HER2 expression status varied significantly in these studies, ranging from quantitative RT-PCR, to FISH/CISH using human probes, to immunohistochemistry with anti-human ERBB2 antibodies. Validation of human-based products has not been performed

in dogs, and a recent publication revealed non-specific binding of human HER2 antibodies in canine mammary tissues.(Burrai et al., 2015) This historical discrepancy, in addition to the more recent reduced prevalence of spontaneous canine mammary tumors following early ovariohysterectomy in the American dog population, has made canine mammary tumors a less pertinent model for human disease in North America.

Spontaneous canine appendicular osteosarcoma is relatively common in comparison to its human counterpart, and holds promise as a model for pediatric osteosarcoma. Controlling metastatic disease is the greatest challenge in treating canine osteosarcoma. While <15% of dogs with osteosarcoma have evidence of metastatic disease at the time of diagnosis, approximately 90% will die of metastatic disease within the first year following amputation alone.(Spodnick et al., 1992) Follow-up with a platinum-based chemotherapy drug prolongs median survival times to 10-12 months,(Berg, Weinstein, Schelling, & Rand, 1992; Bergman et al., 1996) however, there has been little success in developing adjuvant therapies over the last two decades.

HER2 overexpression has been researched as a possible target for immunotherapy in canine osteosarcoma. Studies of expression profiling in humans and dogs have not identified HER2 over-expression as a predominant feature in osteosarcoma.(Milovancev et al., 2013; O'Donoghue et al., 2010; Paoloni et al., 2009; Roy, Wycislo, Pondenis, Fan, & Das, 2017; Scott et al., 2011) Screening of seven canine osteosarcoma cell lines and ten patient tumor samples revealed HER2 overexpression using real-time PCR and IHC in

approximately 40% of cases. (Flint et al., 2004) In this study, a rabbit anti-human polyclonal antibody (Neu C-18, Santa Cruz) was used that has not been validated in dog and is no longer available, and IHC staining was predominantly cytoplasmic. The canine intestinal epithelium sections used as a positive control for IHC also showed cytoplasmic staining in the absence of membranous staining, raising concern for non-specific binding.

The C-18 antibody was reported to show nuclear staining in 34% of mammary carcinomas that was not apparent using the FDA-approved HercepTest antibody.(Schillaci et al., 2012) This pattern suggests either a better ability of the C-18 antibody to detect nuclear and cytoplasmic HER2, or a lack of specificity. Further, in spite of the 93% homology between canine and human ERBB2, western blotting using the HercepTest antibody in canine mammary samples did not yield a band at the expected 185kDa, but instead at two non-specific bands at 50 and 60kDa.(Burrai et al., 2015) These examples of antibody cross-reactivity emphasize the need to validate all antibodies prior to use in new species or settings.

With the possibility of HER2 overexpression in canine osteosarcoma and the success of trastuzumab therapy in human breast cancer, the Listeria-based HER2/neu vaccine was employed as adjuvant therapy following standard-of-care primary disease control and chemotherapy for canine osteosarcoma. In 2016, a phase I clinical trial yielded promising results in providing long-term survival for dogs with osteosarcoma, and the median survival time of the eighteen dogs administered the vaccine was 956 days.(Mason et al., 2016) Inclusion criteria

required that patients have a HER2 positive tumor (defined as >10% of cells staining positive for HER2 on IHC using the same protocol as previously described),(Flint et al., 2004) and to be free of metastatic disease following completion of the standard-of-care chemotherapy protocol (approximately 3 months after diagnosis). The study design raises concern for sampling bias, as dogs with a more aggressive form of osteosarcoma may develop metastatic disease within three months of initiating treatment and are excluded from the study, artificially prolonging survival variables. A large multi-institutional phase III clinical trial evaluating the efficacy of adjuvant treatment with the vaccine compared to standard-of-care treatment is currently underway.

Justification

While the Listeria-based HER2 vaccine shows promise in prolonging survival in dogs with osteosarcoma, there is controversy in defining HER2-positive tumors in dogs, the true prevalence of HER2-positive osteosarcoma in dogs, and the proof of concept of utilizing the vaccine for tumors with cytoplasmic and nuclear accumulation of HER2 when it's efficacy (and the efficacy of the related immunotherapy trastuzumab) has only been demonstrated in tumors with membranous over-expression of HER2.

MATERIALS AND METHODS

Patient specimens and cell culture

Tumor tissue was obtained after surgery or immediately post-mortem from client-owned dogs with spontaneously-occurring OSA at the University of Missouri Veterinary Health Center. Normal colon tissue was obtained post-mortem from patients without intestinal disease euthanized for other reasons from the same institution. All specimens were donated by owners for research purposes (ACUC #8240). Additional fresh frozen OSA specimens were obtained from the Canine Comparative Oncology and Genomics Consortium Biospecimen Repository (Frederick, Maryland). Fresh tissue was snap frozen and stored at -80°C for protein analysis. Tissue was also preserved in 10% formalin and then stored in 70% ethanol.

Cell pellets of HMPOS, Abrams, and PANC-1 were obtained from the University of Missouri Comparative Oncology, Radiobiology, and Epigenetics Laboratory. All cell lines were maintained at 37°C with 5% CO_2 in high glucose Dulbecco's Modified Eagle's Medium (GIBCO, Invitrogen, Carlsbad, California) supplemented with 10% Heat-Inactivated Fetal Bovine Serum and 1% penicillin-streptomycin (GIBCO, Invitrogen, Carlsbad, California), and were stored at -80°C until processed.

Protein identification with mass spectrometry

Two samples were precipitated by adding 5 volumes of cold acetone and incubating at -20C for overnight. The precipitated protein was washed by 80% cold acetone twice. Then 20ul 6M urea 2M thiourea and 100mM ammonium

bicarbonate was added to the pellet. The solubilized protein was reduced by DTT and alkylated by IAA. After that, trypsin was added for digestion overnight. The digested peptide was C18 ziptip desalted, lyophilized and resuspended in 20uL 5/0.1 % acetonitrile/formic acid.

One 1ul suspended peptide was loaded onto a on C18 column (20 cm X 75 µm 1.7 µm) with a step gradient of acetonitrile at 300nL/min. The Bruker nanoElute system is connected to a timsTOF-PRO mass spectrometer (Bruker, Billerica, Massachusetts). LC gradient conditions: Initial conditions were 2%B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by 26 min ramp to 17%B. 17-25%B over 36 min, 25-37%B over 15 min, gradient of 37%B to 80%B over 6 min, hold at 80%B for 7 min. Total run time was 90min. MS data was collected over a m/z range of 100 to 1700. During MS/MS data collection, each TIMS cycle included 1 MS + an average of 10 PASEF MS/MS scans.

Canine raw data was searched using PEAKS (version X) with NCBI canis lupus protein database downloaded on Feb 05, 2019. Human sample was searched with Uniprot Human protein database. Data were searched with trypsin as enzyme, 3 missed cleavages allowed; carbamidomethyl cysteine as a fixed modification; oxidized methionine, acetylation on protein N terminus as variable mod; 50ppm mass tolerance on precursor ions, 0.1Da on fragment ions.

HER2 immunoblotting

Protein was extracted from frozen canine tissue using T-PER (Thermo Fisher Scientific, Waltham, Massachusetts), and from whole cell lysates of

PANC-1, Abrams, and HMPOS cell lines using M-PER (Thermo Fisher Scientific, Waltham, Massachusetts). Protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts).

Samples were heated to 95°C for 5 minutes in Laemmli buffer, and 30 μg of protein per sample was immediately loaded in to NuPAGE 4 to 12% Bis-Tris gels (Invitrogen, Carlsbad, California). Protein was size separated with electrophoresis in MOPS buffer (Invitrogen, Carlsbad, California), then transferred to a 0.45 micron nitrocellulose membrane (Bio-Rad, Hercules, California) in transfer buffer (25mM Tris, 192mM Glycine, 10% v/v methanol, 0.1% SDS). The membrane was blocked at room temperature for one hour in blocking buffer (5% w/v nonfat dry milk,1X TBS, 0.1 % Tween-20), then probed separately with the primary antibodies (recombinant monoclonal rabbit anti-human HER2 IgG [ab214275] at 1:1000 dilution, polyclonal rabbit anti-human β-actin IgG [ab8227] at 1:5000 dilution) (Abcam, Cambridge, Massachusetts) at 4°C overnight. The membrane was incubated for 1 hr at room temperature with a goat anti-rabbit IgG-HRP conjugate (ab6721 at 1:5000 dilution) (Abcam, Cambridge, Massachusetts) secondary antibody.

Following a 5 minute incubation with Luminate Forte Western HRP substrate (Millipore, Billerica, MA), chemiluminescent signal was detected on Kodak Image Station 4000R (Carestream Health, Inc., Rochester, New York) using Kodak Molecular Imaging Software version 5.0.1.17 (Carestream Health, Inc., Rochester, New York). Densitometry was performed using ImageJ software (NIH, Bethesda, Massachusetts). Band densities for total HER2 protein (185

kDa) were normalized to β-actin expression, and were also normalized among experiments using the PANC-1 loaded on all gels.

Histopathology and IHC

Histopathologic examination of H&E-stained formalin-fixed, paraffin-embedded (FFPE) canine osteosarcoma and normal colon tissue sections was performed by a single board-certified pathologist (DYK). IHC for the HER2 protein was used to determine the relative amount and subcellular distribution by the same pathologist. For IHC, following sectioning, specimens were blocked in 3% hydrogen peroxidase (15 min), pre-treated with Borg (Biocare, Pacheco, California), blocked with Sniper (Biocare, Pacheco, California) for 10 min, and incubated with the same anti-HER2 antibody (ab214275 at 1:1000 dilution for 30 min, Abcam, Cambridge, Massachusetts). The intensity of protein staining was scored on a 0-3+ scale (0 = no staining, 1+ = weak staining, 2+ = moderate staining, and 3+ = strong staining). Distribution was described as cytoplasmic and/or membranous.

RESULTS

Antibody validation

We first validated a commercially available anti-human HER2 primary antibody in canine tissue. An anti-human HER2 monoclonal antibody (ab214275, Abcam) targeted against the N-terminal domain of HER2 in human tissue was validated in canine tissue according to manufacturer's directions. HER2 protein expression in the PANC1 cell line, two canine osteosarcoma cell lines (HMPOS and Abrams), normal canine colonic mucosa (positive control), and spontaneously occurring canine OSA was evaluated through immunoblotting and immunohistochemistry. As an established human HER2-positive cell line, PANC1 yielded an expected strong positive band at 185 kDa. Total protein and plasma membrane protein from canine colonic tissue also showed positive immunostaining at 185 kDa and was deemed an appropriate canine positive control (Figure 1). HER2 immunoblotting was also performed on normal canine skin, lung, kidney, liver, and salivary gland yielded only a faint 185 kDa band.

Protein identification with mass spectrometry

Plasma membrane protein extracted from flash frozen fresh canine colon tissue from one of these samples was also submitted for mass spectrometry analysis. The presence of HER2 protein was confirmed via identification of 11 unique peptides (Figures 2-3).

Canine HER2 immunoblotting

This study compared HER2 protein expression in 26 OSA samples (2 cell lines, 24 primary tumors), 2 normal colon tissue positive controls expressing

physiologic amounts of canine HER2 protein, and PANC-1 with the previously validated antibody. In 23 of 26 canine OSA samples, band intensity was two orders of magnitude lower than the human PANC-1 cell line. Canine colon tissue band intensity was approximately half that of PANC-1. No canine OSA samples showed band intensity equal to or greater than those of canine colon tissue or PANC-1 (Figure 4).

Canine HER2 immunohistochemistry

HER2 immunostaining patterns in 6 normal canine colon samples and 18 canine OSA samples (13 primary tumors, 5 metastases), were evaluated by a single board-certified veterinary pathologist, with interpretation of HER2 status according to intensity and cellular localization. Colonic mucosa was positive for membranous immunostaining in all canine colon samples, ranging (++) to (+++) in intensity. Four of 5 OSA metastases and all 13 OSA primary tumors showed some degree of HER2 immunostaining. Six of 13 primary tumors showed HER2 immunostaining intensity comparable to colonic mucosa (++) or greater, and 1 of 5 metastases showed HER2 immunostaining intensity comparable to colonic mucosa (++). Only 1 of all 18 OSA samples showed strong (+++) membranous distribution typical of HER2-positive neoplasms as described in human literature. Examples of tissues and staining patterns are available in Figure 5, and immunostaining pattern and distributions in Table 1 and Figure 6.

Interestingly, pulmonary and renal OSA metastases were available from a patient treated with HER2-targeted immunotherapy, and while the pulmonary lesion demonstrated (++) HER2 immunostaining, the renal lesion showed no (-)

HER2 immunostaining. Colon tissue was available for IHC in this patient as well and showed expected (++) immunostaining.

DISCUSSION

Based upon our findings, only a subset of spontaneously occurring canine osteosarcoma tumors express the tumor associated antigen HER2 in a relevant manner for immunotherapy. Approximate relative HER2 protein expression is comparable (within the same order of magnitude) between HER2-positive canine osteosarcoma tumor samples and normal canine positive control tissue, suggesting poor specificity as a tumor-specific antigen in this disease. In human breast cancer, the classification as a HER2-positive tumor is complicated and controversial. While FISH can demonstrate HER2 gene amplification, not all tumors overexpress HER2 as a result of amplification, and immunohistochemistry can demonstrate expression in these circumstances. Immunostaining can vary significantly based on sample handling and techniques, especially between labs. Strong positive membranous staining only is deemed adequate for HER2-positive classification. In our study, only 1 of 18 tumors met criteria as a HER2 positive tumor.

Following identification of a subset of HER2-positive canine tumors (osteosarcoma, or other) by a qualified board-certified veterinary pathologist using standardized criteria, further research evaluating anti-HER2 targeted therapies in this subset of dogs is warranted. Standardized criteria could include confirmation of appropriate intensity of HER2 immunostaining on IHC, HER2 protein concentrations comparable to human tumors deemed HER2-positive, FISH demonstrating gene amplification (although this has not been demonstrated in dogs).

The paucity of samples showing membranous HER2 distribution in canine osteosarcoma precludes the use of antibody-based therapy. Given that antibodies are large secreted proteins, and their distribution is limited to the extracellular fluid based on their physical characteristics, intracellular sequestration of the HER2 antigen typical of the described cytoplasmic distribution in canine osteosarcoma could interfere with immunorecognition with this approach. Only a subset of canine osteosarcoma tumors may be appropriate for anti-HER2 directed immunotherapy, but not cell surface-targeted therapy.

Anti-HER2 therapies may carry a greater risk for on-target off-tumor cardiotoxic side effects in large and giant breed dogs given their unique concurrent predisposition to the development of both osteosarcoma and dilated cardiomyopathy. The development of dilated cardiomyopathy, systolic dysfunction, and congestive heart failure following anti-HER2 therapy in humans has been well demonstrated, but data are lacking in other species. However, given the indispensable role of HER2 in cardiac and neural development and the similarities between the species, HER2 blockade would likely have similar consequences in dogs.

Challenges

Several challenges were encountered given the nature of a retrospective study, and the quality and availability of previously banked samples. Subcellular fractionation of osteosarcoma samples proved challenging given the amount of tissue needed and mineralized nature of the tissue. Commonly used decalcification techniques can alter protein shape and conformation, resulting in

the potential for a now unrecognizable epitope for primary antibodies used in identification of proteins through immunostaining. This made several historical controls unsuitable for inclusion in this study as previous sample handling would preclude conclusive results.

Future directions

While HER2 expression is limited in canine osteosarcoma, it has also been demonstrated in canine mammary tumors, urothelial carcinoma, and pulmonary adenocarcinoma (Lorch et al., 2019; Seung, Cho, Kim, Lim, & Sur, 2020; Tsuboi et al., 2019). Screening of other tumor types and development of HER2-targeted therapy may benefit canine patients and serve as a translational platform for human therapies.

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Figure 1

Immunoblot using the anti-HER2 antibody and actin loading control in canine colon, colon plasma membrane, HMPOS and Abrams canine osteosarcoma cell lines, and PANC-1 human pancreatic carcinoma cell line

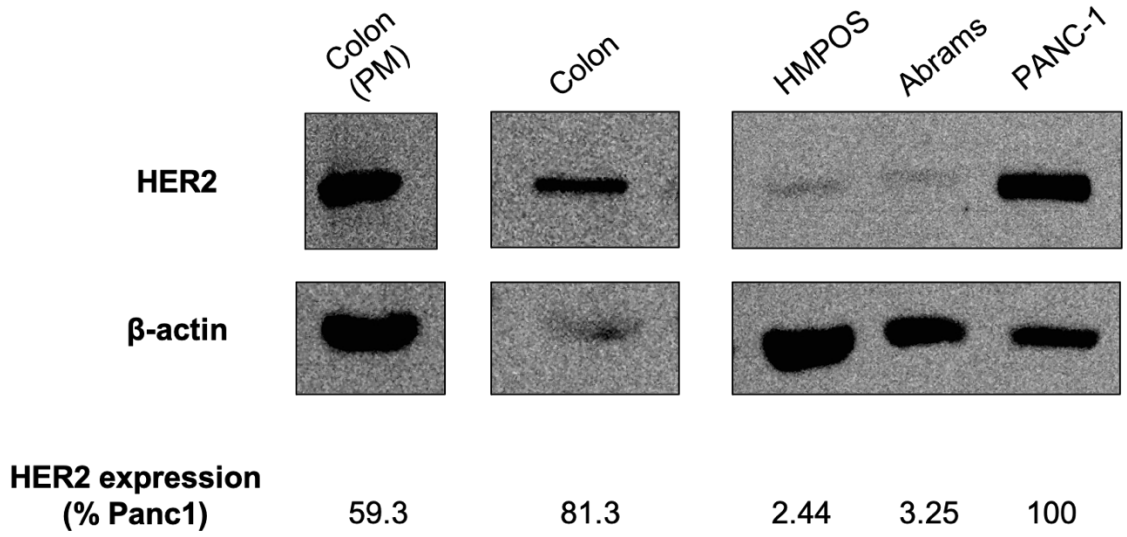
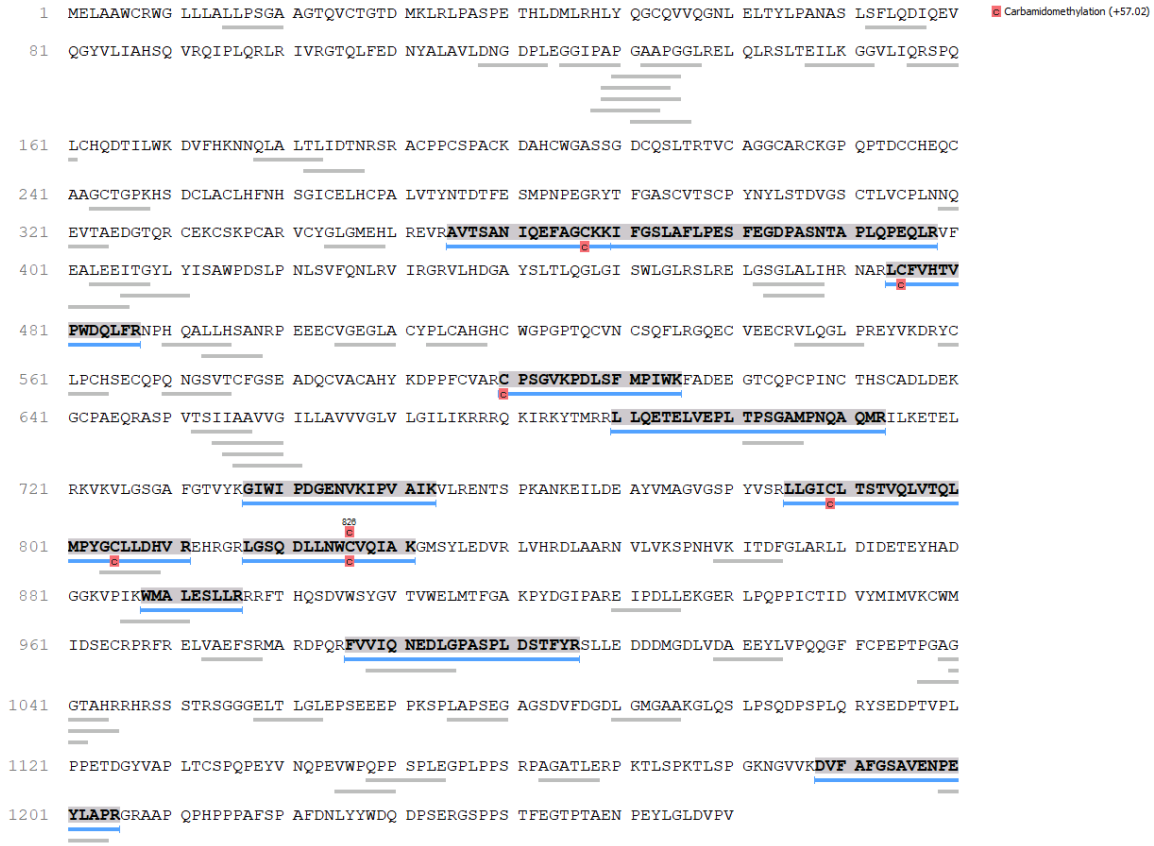


Figure 2

Canine Colon Plasma Membrane HER2 Protein Sequence Coverage



The blue lines represent the peptides identified by MS.

Figure 3

MS Spectra of 3 Peptides from HER2 in Canine Colon Plasma Membrane

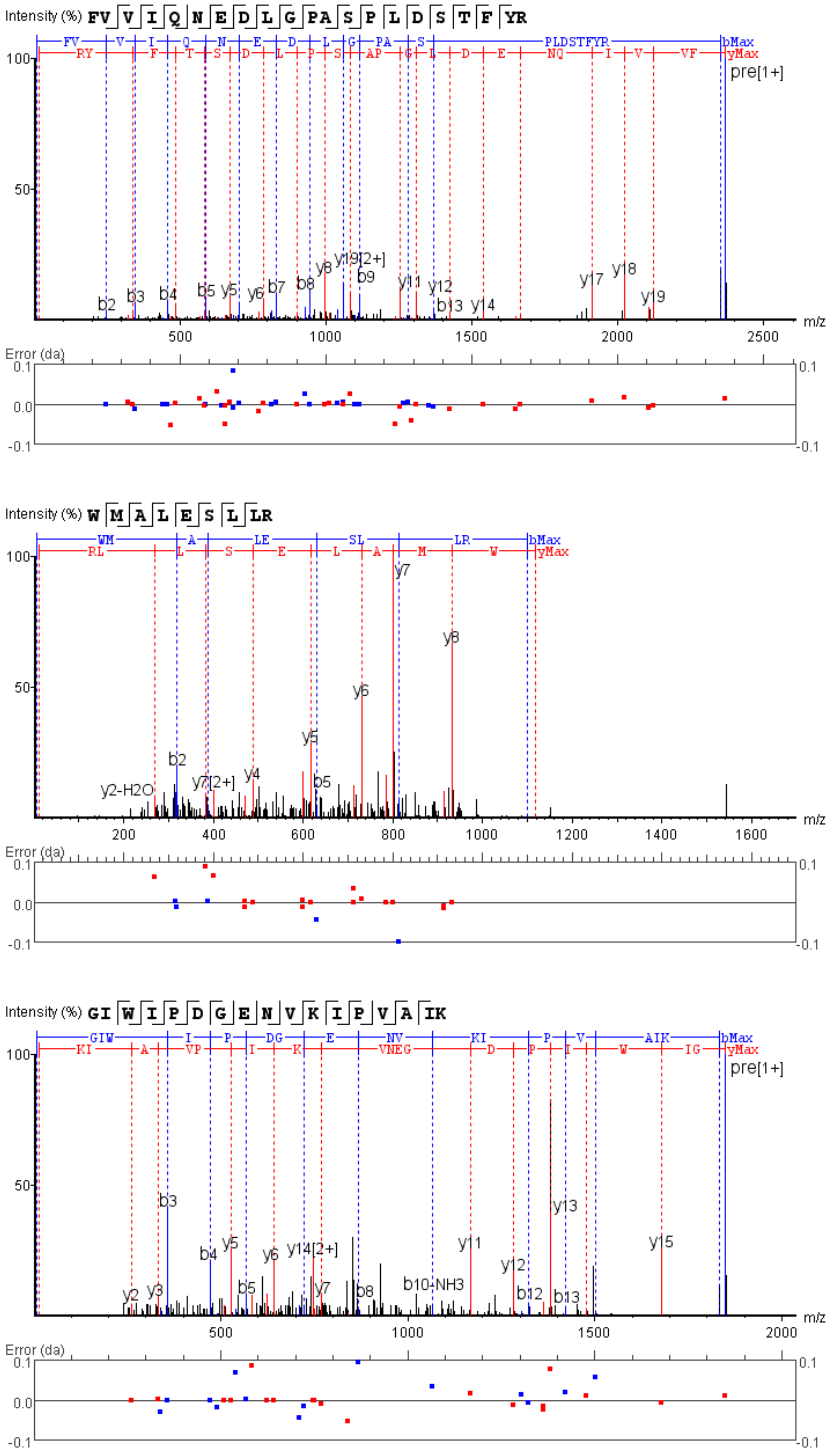
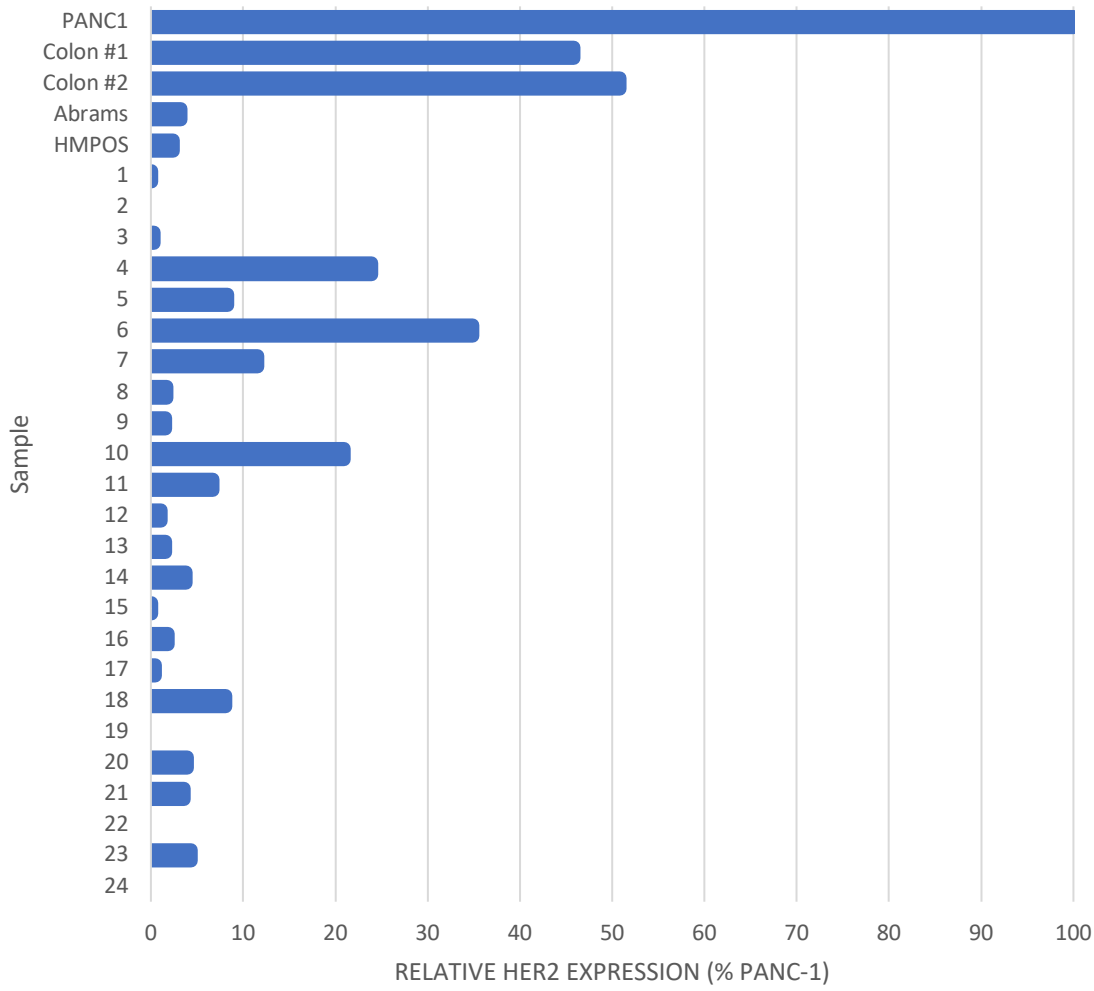


Figure 4

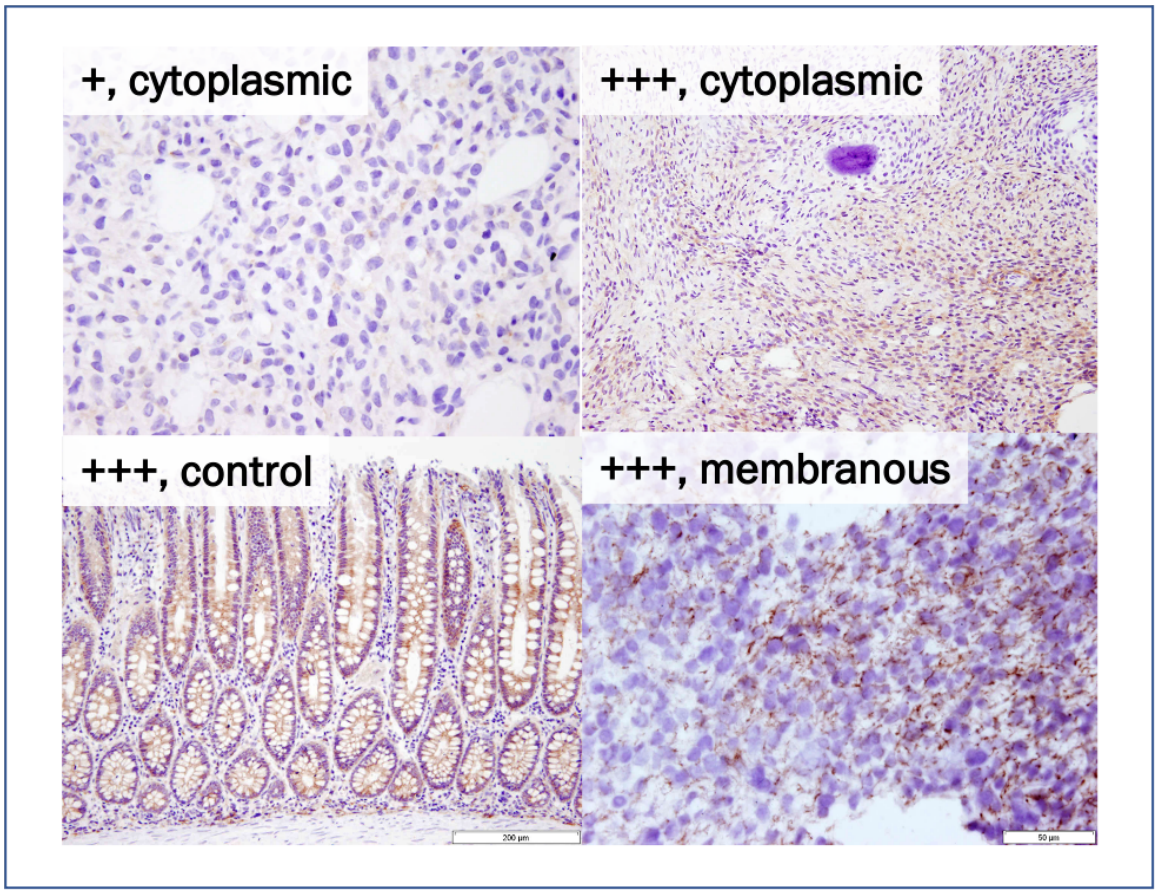
Tumor and Control Relative HER2 Immunoblot Band Intensity



Percent band intensity compared with control (PANC-1) after normalization to the internal loading control (actin) in canine colon tissue, osteosarcoma, osteosarcoma cell lines, and the human PANC-1 cell line.

Figure 5

HER2 Immunohistochemistry of Canine Colon and Osteosarcoma Tissue



Top left: canine osteosarcoma with (+) cytoplasmic staining; top right canine osteosarcoma with (+++) cytoplasmic staining; bottom left canine colon with (+++) cytoplasmic and membranous staining; bottom right canine osteosarcoma with (+++) membranous staining.

Table 1

HER2 Immunoreactivity of Primary and Metastatic Canine Osteosarcoma Tissue

IHC Score	Primary OSA tumors n=13, number (%)	OSA metastases n=4, number (%)
-	0 (0)	2 (50)
+/-	2 (15)	1 (25)
+	3 (23)	1 (25)
++	6 (18)	0 (0)
+++	2 (15)	0 (0)

Figure 6

HER2 immunoreactivity of primary and metastatic canine osteosarcoma tissue