DISSERTATION

INTEGRATED PATHWAYS ASSOCIATED WITH METASTASIS AND CHEMORESISTANCE IN CANINE OSTEOSARCOMA

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ABSTRACT

INTEGRATED PATHWAYS ASSOCIATED WITH METASTASIS AND CHEMORESISTANCE IN CANINE OSTEOSARCOMA

Osteosarcoma (OS) is the most common canine primary bone tumor affecting 10,000 dogs every year. This aggressive cancer is characterized by both a high rate of metastasis and chemotherapeutic resistance. It is estimated up to 80% of patients carry silent metastases at the time of diagnosis, and most will progress despite removal of the primary tumor and chemotherapy. Canine OS is strikingly similar to the disease in humans following a similar clinical course and sharing genetic and molecular aberrations. Thus the canine disease has gained recognition as a relevant spontaneous tumor model for human OS. Unfortunately, survival rates for both species have plateaued with no significant gains made in the last 20-30 years. New treatment strategies are needed and will likely consist of combined therapies including conventional chemotherapy drugs along with targeted and immune modulating agents. The success of clinical trials to evaluate these novel therapies will rely on improved understanding of molecular pathways contributing to progression and chemotherapy resistance of OS. Further, molecular characterization of OS will provide biomarkers essential for prognosis, treatment planning and patient monitoring.

Gene expression profiling of canine tumors from poor responders compared to tumors from good responders implicated pathways critical to normal bone development including hedgehog and Wnt/β-catenin. During bone development, there is significant crosstalk between

these pathways and the Notch signaling pathway, a third developmental pathway associated with growth and survival in a variety of human cancers. We performed pathway focused gene expression studies using canine and human OS cells, canine OS tumors and normal bone samples to evaluate two Notch receptors and two downstream effectors. We identified expression changes consistent with Notch activation in OS compared to normal bone. We further determined that, while expression of three Notch associated genes remained elevated in tumors from the poor responders, expression of hairy/enhancer of split 1 (HES1) was significantly lower in tumors from poor responders than in tumors from good responders. Survival analyses based on immunoreactivity for HES1 in fixed tissues from an independent tumor set confirmed the association between low HES1 expression and poor outcome.

To further explore the Notch pathway in OS and elucidate potential mechanisms underlying the disruption of Notch/HES1 signaling in the most aggressive tumors, we performed miRNA expression profiling of canine tumors. Our goals included identification of miRNA signatures associated with patient outcome in OS as well as integration of miRNA and gene expression data for additional pathway-focused explorations. Further, we endeavored to find miRNA biomarkers in serum of OS patients with prognostic potential. We successfully identified a tumor-based three-miRNA signature and a serum-based two-miRNA signature that separated patients into distinct outcome groups with good accuracy. *In silico* miRNA-mRNA interaction analyses of dysregulated miRNAs and Notch-associated genes in tumors compared to normal bone revealed nearly 20 interactions, validated experimentally in other systems, potentially associated with OS. Interaction and pathway analysis of aberrant miRNA and gene expression in tumors from poor responders vs. good responders identified insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), an oncogene of interest in OS, as a common target

of seven down-regulated miRNAs. Finally, these analyses suggested interactions with the tumor microenvironment are important to the progression of OS.

We expanded our miRNA expression profiling to include microarray analysis of 29 canine cancer cell lines. This allowed us to utilize drug sensitivity data from *in vitro* assays, where cells were treated with either doxorubicin or carboplatin, to identify "drug-resistance-related" miRNAs associated with outcome in canine OS tumors. We identified an additional miRNA from this cell-based approach, which participated in a tumor-based four-miRNA predictive signature. *In silico* miRNA-gene regulatory pathway analyses of outcome associated miRNAs and dysregulated genes from predictive doxorubicin and carboplatin models, developed using the COXEN algorithm, implicated the Notch pathway as contributing to doxorubicin resistance. Finally, pathway analyses of the top five miRNAs associated with progression of OS and chemoresistance: let-7b, miR-98, miR-130a, miR-181b and miR-223 implicated the PI3K/AKT pathway in progression of OS.

Taken together, the studies described herein, provide an integrated picture of Notch signaling in OS elaborating candidate miRNA-gene interactions associated with development and progression of OS and resistance to doxorubicin. Further, these studies have revealed key miRNA-mRNA interactions that implicate other targetable pathways and thus, may serve as biomarkers for patient stratification, enhancing efforts towards integration of individualized targeted therapies in OS. Finally, we have identified miRNA-based prognostic signatures measurable from OS tumors or patient serum, which laid the groundwork for development of a clinically useful prognostic screen for OS.

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DEDICATION

I dedicate this dissertation to my husband, Chad, who is my rock, my best friend, and the most amazing father Calvin could ask for.

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Chapter One

Literature Review

CANINE OSTEOSARCOMA DISEASE REVIEW

Epidemiology

Osteosarcoma (OS) is the most common primary malignant bone tumor in dogs affecting more than 10,000 dogs every year and accounting for greater than 85% of canine skeletal malignancies.[1, 2] Canine OS typically occurs in middle-aged to older dogs (median age ~7 years), but also rarely affects dogs at 18-24 months of age.[3] Large or giant breed dogs are most commonly affected with fewer than 5% of OS arising in dogs weighing less than 30 pounds.[1, 4] Size appears to be a stronger risk factor than breed; however, increased risk has been demonstrated for Saint Bernards, Great Danes, Irish wolfhounds, Doberman pinschers, Rottweilers, German shepherds and golden retrievers.[2-7] Further, there is growing evidence for heritability of OS in Scottish deerhounds, Rottweilers, greyhounds, Irish wolfhounds and Saint Bernards.[8-11] Males are reported to be affected slightly more commonly than females, and at least one report has associated gonadectomy with increased risk for OS.[1, 3, 12] Approximately 75% of tumors affect the appendicular skeleton with the forelimb affected two times more often than the hindlimb; the most common tumor locations include the proximal humerus, distal radius, distal gemur, distal tibia, and proximal tibia.[2, 3, 13, 14] The most commonly affected sites in the axial skeleton are the mandible and maxilla.[13]

Clinical Presentation, Pathology, and Prognosis

Dogs with OS typically present with lameness and swelling at the site of the primary tumor, with or without a history of trauma or evidence of fracture. Patients with radiographic evidence of pulmonary metastasis may have non-specific signs of systemic disease such as loss of appetite and general discomfort, but rarely have respiratory signs.[1] Radiographic lesions are highly variable, but typically include both lytic and osteogenic features including cortical lysis and extension into adjacent soft tissue. OS lesions do not directly cross articular surfaces. The primary non-malignant differential on radiographs is fungal osteomyelitis.[15] Histologic appearance of OS tumors is also highly variable both between and within tumors including pleomorphic tumor cells and variable a mounts of osseous, cartilaginous or fibrous matrix. Production of poorly defined woven bone or "osteoid" by tumor cells is the definitive pathologic criteria for diagnosis differentiating OS from other sarcomas such as chondrosarcoma or fibrosarcoma. Staining of cytology specimens for alkaline phosphatase (ALP) may also be useful for distinguishing OS from other tumor types.[16-18]

The presence of different types of matrix material and other morphologic features in OS tumors may be used to classify tumors into osteoblastic, chondroblastic, fibroblastic, telangiectatic and giant cell types. Unfortunately, no significant difference in patient outcome has been established for the most common subtypes.[1, 19] Two histologic grading schemes have been proposed for canine OS and various studies have found associations between grade and outcome, though neither scoring system has gained wide acceptance in the veterinary pathology or oncology communities.[20, 21] This is likely due to a combination of factors including the variability within tumors as well as the complexity of the criteria in the proposed grading

schemes. Each of these aspects of tumor grading contributes to high subjectivity. Further, more than 80% of tumors will fall into high-grade histologic categories, within which variable patient outcomes may be achieved.[1, 22] Other prognostic clinical factors consistently associated with decreased survival or shorter disease free interval (DFI) include proximal humerus location,[1, 23, 24] increased body weight,[23] elevated serum ALP,[23-25] and clinical evidence of pulmonary or lymph node metastasis at time of diagnosis.[3, 26] Some studies have also identified association between increased age,[6, 24] increased lymphocyte count, increased monocyte count,[27] and tumor size.[3]

Metastasis and Treatment

Canine OS is a highly malignant sarcoma commonly metastasizing to the lungs and bones, and rarely local lymph nodes.[1, 19, 28] Though fewer than 15% of canine patients present with radiographic evidence of pulmonary metastases, up to 90% will ultimately succumb to metastatic disease if amputation, the most common treatment of local disease, is the only treatment.[29] Current standard of care treatment, therefore includes surgery, either amputation or limb-sparing tumor excision, combined with adjuvant chemotherapy, most commonly doxorubicin and/or platinum-based drugs.[1] The addition of systemic therapy has increased the median survival time (MST) in canine patients from around 120 days to 250-300 days.[1, 23] Numerous studies have been conducted evaluating different combinations or dosing regimens of cytotoxic drugs, but a recent study by Selmic et al found that in 470 dogs, choice of protocols did not result in significantly different survival times.[23] Subsequently, the focus of current research has shifted to identification and evaluation of immunomodulatory and targeted

molecular drugs, which might result in improved survival when used in combination with cytotoxic chemotherapies. Clinical trials in dogs have evaluated these types of compounds including muramyl tripeptie phosphatidylethanolamine (L-MTP-E), a somatostatin analog targeting growth hormone (GH) and insulin-like growth factor one (IGF-1), and an inhibitor of matrix metallopretinases (MMPs). Thus far all have shown inconsistent or disappointing results rarely demonstrating any clinical advantage over treatment with chemotherapy alone.[30-33]

COMPARATIVE ASPECTS OF OS

Clinicopathologic Factors and Prognosis

OS is also the most common primary bone tumor affecting human adolescents age 10-20 years.[34] There is remarkable similarity between pediatric osteosarcoma and the presentation and clinical course of the canine disease. These similarities have been extensively reviewed and include radiographic changes, predominance of high histologic grade tumors, rate and sites of metastases, response to chemotherapy and shared risk factors of increased body weight, alkaline phosphatase levels and evidence of pulmonary or lymph node metastasis. [1, 22, 35-37] One noteworthy difference is that OS occurs less frequently in humans affecting approximately 1000 children or adolescents per year.[1, 34] In both species the age of onset follows a bimodal distribution; however, juvenile onset is rare in canines and later onset in humans is typically associated with Paget's disease. Thus the most common spontaneous presentation occurs in older dogs and younger humans.[3, 34] The most common skeletal locations are similar in dogs and humans with tumors typically arising in metaphyseal regions of long bones of appendicular

skeleton. In dogs the forelimb is most frequently affected at the proximal humerus or distal radius, while the most common human lesions affect either the femur or the tibia near the knee. One hypothesis for this difference is that tumors are more likely to arise in the primary weight bearing long bones, which are the forelimbs in dogs and the legs in humans.[3, 14, 34] Treatment of OS is similar in dogs and humans including surgical resection of the primary tumor and chemotherapy including doxorubicin and platinum based drugs to treat systemic disease assumed present in up to 80% of patients. Human protocols more commonly include additional drugs including methotrexate and ifosfamide. The most significant difference in treatment is that human patients are routinely treated with pre-adjuvant chemotherapy.[38, 39]

Response to pre-surgical chemotherapy via pathologic assessment of post-treatment tumor necrosis is one of the most predictive clinical determinants of overall patient outcome in human OS.[34, 39] Other consistent prognostic factors are similar to those already discussed for canine patients including large tumor size, increased serum ALP and lymph node involvement.[34, 39-41] No significant clinical benefit has yet been demonstrated for pre-surgical chemotherapy in canine patients.[1, 42, 43] One recent report suggested neoadjuvant treatment with intratumoral Fas ligand gene therapy led to an inflammatory response associated with increased survival. [44] Nonetheless, pre-surgical treatment is not presently widely practiced for canine patients. The availability of drug naïve canine tissues and the increased incidence of OS in dogs contribute to the value of the dog as a comparative model in OS research.

Unfortunately, the similarities between human and canine OS extend to the stagnation of cure rates. Despite increased disease free survival from less than 20% to 55-75% with the addition of pre- and post-operative chemotherapy, the cure rate for human OS has not altered

much in the last thirty years.[38] Further, patients presenting with metastasis either at time of diagnosis or after standard of care treatment continue to have a grave prognosis.[38] Current research for both species therefore has focused on improved chemotherapy agents and strategies including identification and incorporation of targeted therapies and individualized treatment based on molecular profiles. Even before publication of the human and canine genomes and the explosion of genomic-based research, the value of spontaneous canine OS as a model of the human disease was recognized.[35, 45] Newer reports clearly demonstrated that in addition to clinicopathologic similarities, canine and human OS are indistinguishable on a molecular level.[36, 46-49] Thus ensuing discussions regarding significant genetic factors and molecular aberrations contributing to the pathogenesis of OS will review evidence in the literature from both species.

Genetic Factors

The etiology of OS remains elusive, though a variety of host, environmental and genetic factors likely contribute to OS pathogenesis. Genetic changes in OS are comprised of a complex karyotype with numerous, and often inconsistent, chromosomal aberrations. For example, in a 2003 review, Sandberg et al included a table spanning 6 pages listing chromosomal aberrations found in human OS.[50] This is in contrast to many human sarcomas that involve consistently recurring and specific genetic changes, such as the well-established EWSR1-FLI translocation in Ewing's sarcoma. Many of the most common of these chromosomal changes also occur in canine OS.[51, 52] Multiple reviews of the genomic instability inherent in OS have hypothesized the recently described phenomenon of chromothripsis, a single catastrophic event leading to

hundreds of genomic rearrangements, as a potential underlying mechanism.[53-55] Several studies have found changes in mechanisms responsible for the maintenance of telomeres, DNA sequences associated with chromosomal stability, occur in OS.[56, 57] Telomerase activity (TA), responsible for enzymatic lengthening of telomeres, is reportedly low in OS with conflicting reports of association between TA and outcome.[58, 59] An increase in a second mechanism for maintenance of telomeres, alternative lengthening of telomeres (ALT), reportedly occurs with increased frequency in OS compared to other cancers and is associated with chromosomal instability.[57, 60]

Though no specific, single, recurrent chromosomal aberration has been identified as central to osteosarcomagenesis, mutations resulting in loss of prominent tumor suppressors and oncogenes occur in both human and canine OS. A handful of these disrupted proteins are responsible for maintaining cell cycle checkpoints and their loss may further contribute to the genetic instability of OS tumors. Approximately 60-70% of human OS tumors demonstrate loss of heterozygosity (LOH) at 13q14, the locus containing the retinoblastoma gene (RB1).[50, 53] LOH of 13q14 in the germ line comprises one of the earliest identified inherited cancers resulting in malignant ocular tumors of affected children.[61] Further, children with the heritable form of retinoblastoma (Rb) experience increased risk of secondary tumors including OS.[62] In addition to loss of RB1, inactivating mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A) and amplification of cyclin-dependent kinase 4 (CDK4), genes coding for regulatory proteins of the Rb pathway, have also have been identified in OS.[54, 63] LOH or deletion of the RB1 is not common in canine OS, but RB1 copy number loss has been documented in 29% of cases in one study.[51, 64] Similar to findings in human OS, copy number imbalances occurred

in CDKN2A and CDK4, and if grouped with loss of RB1 suggests dysregulation of the Rb pathway may occur in put o 45% of affectd dogs.[51, 65]

A second pathway frequently affected in OS and associated with protecting the genome is the p53 pathway. In humans tumor protein p53 (TP53) is mutated in 20-50% of high grade OS. Mutations and amplifications are also reported in p53 pathway related genes including mouse double minute 2 homolog (MDM2), checkpoint kinase 2 (CHEK2) and cyclin dependent kinase inhibitor 2 (CDKN2 or p14ARF).[13, 53, 54] Similar to the case with the Rb pathway, germ line mutations in TP53 manifest as the Li Fraumeni syndrome, with sufferers experiencing increased risk of OS.[53, 66] TP53 mutations have also been reported in up to 40% of canine OS tumors and TP53 mutations were associated with shorter median survival time in one study.[67, 68]

In canine tissues, Thomas et al found copy number losses of another tumor suppressor, phosphatase and tensin homolog (PTEN) in 41% of tumors, making it the most common target of copy number losses in their study.[51] Previously this same group identified mutations of PTEN in 4/5 canine cell lines and correlation between mutations of PTEN and negative or variable protein staining in OS tumors.[69] PTEN loss has also been reported in tumors from several breeds of dogs with increased risk of OS.[51, 52] Though not as common as RB1 or P53 alterations in human OS, 15% of 275 human tumors have biallelic deletion at PTEN locus and up to 33% have monoallelic deletion of PTEN. Further, biallelic deletion of PTEN correlated significantly with negative or weak PTEN protein expression via IHC, though there was no association between PTEN loss and any clinical parameters.[70] In addition to tumor suppressors, amplifications of oncogenes including v-myc avian myelocytomatosis viral oncogene homolog (MYC) and (runt related transcription factor2) RUNX2 have been demonstrated in both human and canine OS.[51, 52, 71] For MYC, amplification did not

translate to increased gene expression in canine tumors leaving the role of MYC amplification in OS tumors unanswered.[52] RUNX2 on the other hand is over-expressed in OS in both species and high expression correlates with poor response to chemotherapy in humans.[65, 72] The genetic changes described provide insight into potential underlying pathways contributing to OS, but only occasionally provide prognostic information and most are not accessible targets for drug intervention.

Molecular factors and biomarkers

A handful of genetic changes described above result in expression changes associated with outcome. Gene expression changes may also occur due to epigenetic or post-transcriptional mechanisms. Additionally, in the case of cell signaling receptors, aberrations of expression may involve autocrine loops, dimerization with other dysregulated receptors or paracrine communications. A growing body of literature has grown out of the need to characterize molecular changes responsible for disease progression, particularly metastasis, in OS. One major objective for these studies is to identify targetable cellular mechanisms responsible for malignant phenotype in cancer cells. In a 2011 review of human OS, Hameed and colleagues discussed promising targeted therapies under investigation in clinical trials in human OS that have resulted from molecular biomarker discoveries.[39] Many targets of these potential therapies have also been explored in canine OS.[73]

Several tyrosine kinase receptors (RTKs) or their ligands, responsible for activating intracellular pathways involved in proliferation and cell survival are expressed in OS. Some of these are well-studied in both human and canine OS include growth hormone (GH)/IGF1R[74,

75], erb-b2 receptor tyrosine kinase 2 (ERBB2 or HER-2/neu) [76], platelet-derived growth factor receptor (PDGFR) [77, 78], hepatocyte growth factor (HGF)/MET proto-oncogene, receptor tyrosine kinase (MET) and epidermal growth factor receptor (EGFR).[17,41,49] Kinase insert domain receptor (KDR or VEGFR), another RTK involved specifically in angiogenesis and implicated in human OS, has been found increased in canine OS secondary to other activated oncogenes including signal transducer and activator of transcription 3 (STAT3).[79] Another pathway associated with proliferation that is activated in OS in both species downstream of several of these RTKs is mTOR.[80] Of these, IGF1R, PDGFR, mechanistic target of rapamycin (MTOR), MET and EGFR inhibitors were evaluated *in vitro* or *in vivo* in canine cell models of OS with promising results.[33, 77, 80-82] Unfortunately, IGF1-R inhibition via a somatostatin analogue, the only one evaluated in a canine clinical trial, failed to show benefit over treatment with standard of care chemotherapy alone.[33] PDGFR protein expression in human tumors and VEGF protein levels in canine serum associated significantly with disease free survival.[78, 83] While expression of MET failed to correlate with outcome in one canine study, a significant association between MET mRNA levels and lymph node metastasis was identified.[84] Other proteins associated with inhibition of apoptosis and with tumor-extracellular matrix interactions that have been implicated in OS are survivin and ezrin.[85-87] Both have associations with poor outcome in canine patients and confer pro-growth and survival effects that were modified by targeted pharmacologic inhibition. A recent meta-analysis confirmed an association with high ezrin protein expression and both recurrence rate and survival in human OS patients.[88]

Given the molecular and genetic heterogeneity in osteosarcoma cells and tumors, identifying a single gene driving disease progression in OS is unlikely. Thus, numerous groups have applied high throughput genomic methods to biomarker and novel target discovery in OS,

including three studies utilizing canine tissues. [89-91] In 2009 a group from the Netherlands used a "home made" canine specific cDNA microarray to measure expression of over 20,0000 genes in two cohorts of 16 tumors each. They found 51 differentially expressed (DE) genes, all over-expressed in the cohort surviving less than 6 months after initial diagnosis. Pathway analysis using the genes in this study and compared to results of similar analysis in human studies identified enrichment for two major pathways: Wnt/β-catenin and chemokine/cytokine signaling.[90] A study conducted two years later took a different approach using a commercially available canine cDNA microarray to measure gene expression in 26 primary cell cultures derived from patient tumors. Unsupervised hierarchical clustering separated the samples into two distinct groups characterized by differential expression of 282 DE genes. Survival was significantly shorter in the group whose gene expression profile showed overexpression of a "G2/M transition and DNA damage" gene signature. Interestingly they were able to use the 282-gene signature to successfully segregate groups of independent canine and human tumor into the two clinically relevant groups with moderate success.[89]

In 2010, our laboratory utilized the same commercial microarray to measure gene expression in two cohorts of tumors based on disease free interval (DFI) following standard of care treatment. Twenty-eight genes were commonly DE in tumors from dogs with DFI < 100 days compared to tumors from dogs with DFI > 300 days using two commonly cited array normalization algorithms; DE of 15 of these was validated via RT-qPCR. Subsequent pathway analysis with validated genes identified enrichment of oxidative phosphorylation, Hedgehog (HH) and parathyroid hormone (PTH) signaling, immune responses, and for one normalization method, Wnt/β-catenin signaling.[91] Both the 2009 Selvarajah study and our study sought to identify gene expression changes associated with a metastatic and/or chemoresistant phenotype

by comparing tumors from different outcome groups. It is noteworthy that both studies identified enrichment of developmental pathways important for normal bone growth often hijacked in cancer, namely Wnt/β-catenin signaling and hedgehog (HH)/parathyroid (PTH) signaling.[90, 91]

NOTCH AND OS

There is evidence for disruption of both Wnt/β-catenin and hedgehog signaling in the human literature.[92-94] Wnt/β-catenin and hedgehog along with fibroblast growth factor (FGF), TGFβ/BMP, and Notch are five conserved pathways comprising the stem cell signaling network and responsible for the delicate balance between proliferation and differentiation in developing cells. Several reviews have described crosstalk among all five signaling cascades necessary to maintain the homeostasis of stem cells, that, when disrupted, contributes to malignancy.[95-99] In literature elucidating the role of Wnt/β-catenin in both normal bone development an in OS, significant cross-talk between Wnt/β-catenin and Notch is described.[100, 101] Additionally, a recent study by Ma and colleagues demonstrated that inhibition of both pathways synergistically enhanced sensitivity of human OS cells to methotrexate, further implicating both pathways in OS pathogenesis.[102]

During normal bone development, Notch activity is important for maintaining a population of committed osteoblast precursors in the bone marrow, while inhibiting terminal differentiation of osteoblasts.[103-105] Notch and expression of the downstream effector, HES1, demonstrate an oncogenic role in T-cell acute lymphoblastic leukemia (ALL), ovarian, breast, cervical, prostate, colon and non-small cell lung cancers.[106-109] In contrast, Notch activity has

been associated with tumor suppressor behavior in B-cell ALL, myeloid leukemia, hepatocellular carcinoma and neuroblastoma.[110-113] In human OS, Notch and hairy/enhancer of split 1(HES1) expression studies largely suggest an oncogenic role for Notch activity in OS. Engin, from Howard Hughes Medical Center, and Tanaka, out of Japan, published reports connecting Notch activity to a proliferative response in OS, including regulation of cell cycle proteins.[114, 115] Hughes and colleagues at MD Anderson, on the other hand, published reports suggesting Notch, specifically the NOTCH1 receptor and HES1, were involved in metastatic capabilities of OS cells and associated with poor outcome.[116, 117] One of the Hughes' publications was later retracted due to the fact that some of the cells used in their experiments were found to be colon cancer, not OS cells. Interestingly in all of the studies cited above, expression of Notch receptors and effectors was variable across tumor samples and cell lines, with effectiveness of inhibition of Notch to mediate proliferation or migration and invasion also varying. In a recent review, the Hughes lab acknowledged the complexity of Notch signaling in OS and other cancers and switched their focus to Notch signaling as a regulator of angiogenesis contributing to OS metastases.[118] In Chapter 2, we explore expression of Notch signaling components NOTCH1, NOTCH2, HES1 and hairy/enhancer of split related with YRPW motif 1 (HEY1) and the association of both gene and protein expression of HES1 and outcome in canine OS tumors.

In our studies exploring expression of Notch associated genes in OS, we identified a potential disconnect between expression of HES1 and expression of other Notch receptors and effectors in the most aggressive canine OS tumors.[119] Our efforts turned toward identification of post-transcription factors that might influence HES1 gene or protein expression. A study focusing on neuronal development suggested post-transcriptional regulation of HES1 via the microRNA, miR-9, was essential for maintenance of neural stem cells.[120] Subsequently, miR-

9 expression was found upregulated in 19 OS cells compared to normal bone in one study[121] and the HES1-miR9 interaction was identified as part of an important miRNA-transcription factor co-regulatory network associated with proliferation of OS cells by Poos et al.[122] Another gene identified in earlier studies in our lab as highly expressed in aggressive OS, insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), is an oncofetal protein regulated by the well-characterized let-7 family of tumor suppressor miRNAs. A prominent review of miRNA-gene regulatory networks commonly involved in cancer demonstrated let-7 target genes including IGF2BP1 and N-myc interact to regulate proliferative and metabolic pathways in embryogenesis and cancer.[123] We became interested in further exploration of miRNA expression in canine OS to augment biomarker discovery and novel therapeutic target discovery efforts underway at the Flint Animal Cancer Center at Colorado State University.

MICRORNA BIOGENESIS

MicroRNAs (miRNA) are small endogenous RNAs approximately 22 nucleotides long that do not code for protein, but are involved in post-transcriptional gene regulation. Victor Ambrose and colleagues identified the first miRNA, lin-4, a gene involved in the timing of progression from one larval stage to the next in *C. Elegans*.[124] They identified two forms of the lin-4 gene, one 22 and one 61 nucleotides long, the longer proposed to be a precursor of the shorter, with antisense complementarity to lin-14, a second gene involved in *C. Elegans* whose expression was down-regulated via lin-4. Gary Ruvkun's group further demonstrated that regulation of lin-14 via binding of lin-4 in the 3' UTR region resulted in decreased protein expression of LIN-14 without changes in mRNA expression.[125] By 2000, the Ruvkun lab

identified a second miRNA, let-7, involved in *C. Elegans* development and also highly conserved in sequence and function across diverse species including mollusks, arthropods and vertebrates including mice and humans. [126-128] Within a year, 15 microRNAs were identified in *C. Elegans*, some with involvement in processes beyond larval development.[129] By 2003, the number of miRNAs in *C. Elegans* neared 100, many of which are included in the growing list of hundreds of miRNAs conserved in vertebrates and non-vertebrates.[130-132] Additionally, miRNAs are grouped in families of closely related miRNAs redundant in nucleotides 2-8 on the 5' arm. This "seed sequence" is the region typically binding to the 3' UTR of target messenger RNAs (mRNAs) leading to subsequent down-regulation of protein expression of the targeted mRNA.[130, 131, 133] MiRNAs in mammals alone are estimated to regulate 30 – 80% of human genes, most involved in development, transcription or transcriptional regulation, but with more diversity of function than that identified in plants and invertebrates.[133]

A database of identified miRNAs, miRBase (version 21, June 2014), currently lists 2603 mature human miRNAs, many highly conserved between a surprising diversity of organisms; for example, at least half of miRNAs found in *C. Elegans* are homologous in humans[134]. Most miRNAs are located distant from protein coding genes, but up to 40% are intergenic, found in introns, in the same orientation as mRNAs, and processed directly from introns as they are spliced. Up to 50% of miRNAs are clustered together with evidence that they are transcribed as a polycistronic unit.[135] The majority of miRNAs are transcribed by Pol II forming a primary transcript several kilobases long (pri-miRNA)[135, 136]. Evidence suggests the pri-miRNA transcript is processed co-transciptionally,[137] cleaved by the RNAse III enzyme Drosha to produce a stem loop precursor (pre-miRNA)[138]. In animals, Drosha and co-regulatory proteins including DCGR8, form the microprocessor complex responsible for pri-miRNA

processing[139, 140]. The endogenous hairpin-shaped pre-miRNA transcript is exported from the nucleus by exportin 5 (EXP5)[141, 142]. In 2001, Grishok et al. showed that, in the cytoplasm, the two *C. Elegans* miRNAs, lin-4 and let-7 were processed by the same machinery responsible for RNA inhibition (RNAi); others in the field confirmed the role of Dicer, another RNAse III enzyme, in miRNA processing[143, 144] [145]. Interactions between Dicer and TAR (HIV-1) RNA binding protein 2 (TARBP2 or TRBP) lead to the recruitment of Ago2 and serve as a platform for assembly and loading of the mature miRNA into the RNA-induced silencing complex (RISC)[146-149].

In mammals, Ago2 and GW182 comprise the primary functional unit of the RISC. Association of the double-stranded miRNA with Ago 2 leads to rapid strand unwinding and binding of the mature single-stranded RNA in a conformation that exposes the seed sequence nucleotides for Watson-Crick binding to target mRNAs.[150-152] The complementarity of this binding is the primary determinant of the method of gene silencing.[146, 150, 153, 154] Complete or near complete complementarity facilitates Ago2 catalyzed cleavage resulting in mRNA degradation.[154, 155] In mammals, it is more common for the binding to have incomplete complementarity, with central mismatches preventing Ago2-directed cleavage, but instead promoting repression of translation.[150, 156] Carthew et al summarized the three leading proposed mechanisms behind translational repression[150]: 1) repression of 5' cap recognition due to competition between the miRNA RISC (miRISC) and eIF4E (a factor required for translation initiation)[157-159], 2) miRISC mediated deadenylation of the mRNA tail[160-163] and 3) inhibition of association of the 60S ribosomal subunit with the 40S preinitiation complex by miRISC[164, 165]. In the second model of mRNA repression, there is degradation secondary to deadenylation, decapping and exonucleolytic digestion, and not Ago-

catalyzed cleavage [Bagga 2005, Lim 2005]. Regardless of the mechanism, the majority of miRNA-mRNA interactions result in inhibition of expression of the target gene. Given that approximately half of mammalian genes maintain miRNA-binding sites in the 3' UTR, it is not surprising that miRNAs function to fine tune and contribute to the overall robustness of a wide variety of biological processes.[151, 166]

MICRORNAS AND CANCER

Given the role of miRNAs in gene regulation during development, directing cell processes including differentiation and proliferation, it is not surprising that dysregulation of miRNAs is associated with cancer. In 2002, long before the mechanisms of miRNA suppression of gene expression were fully elucidated, Croce's lab published the first evidence for involvement of miRNAs in human cancer.[167] Their work identified that *miR-15a* and *miR-16a* were located within the 13q14 "tumor suppressor" locus frequently lost in chronic lymphocytic leukemia. Within two years, Croce's group published two more reports identifying other miRNAs differentially expressed in B-cell chronic lymphocytic leukemias and proposing that over half of the miRNAs known at that time were located in genomic regions of deletion, amplification or breakpoints associated with several human malignancies.[168, 169] In addition to genomic changes affecting miRNA expression, other groups found evidence of post-translational expression changes associated with various tumors such as loss of miRs-143 and -145 in colorectal cancer[170] and loss of let-7a in lung cancer.[171, 172] These two early studies of miRNA dysregulation in lung cancer were each seminal reports. Takamizawa et al. were among the earliest to show that miRNA expression changes were associated with outcome,

identifying that loss of let-7a was predictive of shorter post-operative survival.[172] Johnson et al from the Slack laboratory demonstrated regulation of RAS, a well-established oncogene often activated in human cancers, by the let-7 miRNA family in HepG2 cells and established an association between let-7 expression levels and RAS protein levels in lung cancer.[171] Slack's group would later identify a KRAS variant consisting of a single nucleotide polymorphism (SNP) in the 3' UTR of KRAS associated with increased risk of non-small cell lung cancer and in seven of the 60 human cancer cells that make up the NCI-60 cell line panel.[173, 174]

In 2005 Lu et al utilized a bead-based hybridization method to measure expression of 217 mammalian miRNAs in 334 samples including a variety of tumors and normal tissue. They documented that overall, miRNAs were largely down-regulated in tumors compared to normal tissue underscoring the importance of miRNA in controlling normal cell growth.[175] This theme is also supported by the finding that mutations of the 3' UTR of target genes, such as the SNP identified in KRAS by the Slack laboratory, occur frequently in cancer allowing cancer cells to escape miRNA regulation.[176, 177] In fact, one group has created a database of somatic mutations that may create or disrupt miRNA targeting including mutations in both miRNAs and target genes experimentally validated to have functional consequences associated with cancer.[178] In addition, Mayr and Bartel showed that alternative cleavage and polyadenylation resulting in shortening of regulatory 3' UTRs represents another mechanism of escape from miRNA regulation that is pervasive in oncogenes in cancer cells.[179] Finally, mutations and expression changes in genes associated with processing of miRNAs also occur in cancer. Examples include down-regulation of Dicer in non-small cell lung cancer[180] loss of argonaute genes in Wilms tumors and testicular germ cell cancers[181, 182], and mutations in exportin-5 to trap miRNAs in the nucleus of colon, stomach and endometrial cancers.[183]

Consistent with the findings of Lu[175] that miRNA expression is generally lower in tumors, most of the early cancer-related miRNA expression studies identified miRNAs with tumor suppressive roles, where loss of miRNA expression is associated with increased expression of various oncogenes and disease. Eis et al was one of the first reports of increased miRNA expression associated with disease, specifically miR-155 in diffuse large B cell lymphoma (DLBCL). This study was also among the earliest to utilize miRNA expression to classify tumor subtypes showing high levels of miR-155 in the more aggressive activated B-cell (ABC) phenotype of DLBCL compared to the germinal center (GC) phenotype.[184] The same year another group identified overexpression of miR-21 in glioblastoma and presented evidence that this oncogenic miRNA blocked apoptosis.[185] In the next 10 years, investigations into miRNA expression changes associated with cancer and functional studies confirming target genes would reveal that miRNAs could act as either oncogenes or tumor suppressors depending upon the function of the genes they inhibit. "Oncomirs", a term originally used for any miRNA associated with cancer[186], would become widely used specifically for miRNAs purportedly acting as oncogenes.[187]

Additionally, although some miRNAs, such as the let-7 family and miR-21 predominantly function as tumor suppressors or oncogenes, respectively, in the majority of cancers other miRNAs may act as an oncogene in one tumor type and a tumor suppressor in another.[186, 188] This is not surprising given the history of intricate temporal and tissue-specific miRNA expression described during development. The miR-200 family, a group of miRNAs established as negative regulators of the epithelial mesenchymal transition (EMT) during both development and cancer[189, 190], provide an interesting example of a miRNA family with potentially different roles depending on both tumor subtype and stage of disease.

Low expression of miRNA 200 family members in the basal-like subtype of breast cancer is associated with poor outcomes; however, high expression of miRNA 200 has been identified in breast cancer metastases and is thought to drive the metastatic phenotype, especially in estrogen receptor positive (ER+) tumors.[123, 191, 192] The role of promoting metastasis is attributed to gene targets not necessarily regulated by miR-200 during EMT.[193] In a 2005 study Chen and colleagues inhibited a number of miRNAs to evaluate their effect on growth and apoptosis of two different cultured cancer cells (cervical and lung). Their report includes miRNAs, such as miR-24, that increased the growth of HeLa cells, but slowed the growth of A549 cells, suggesting tissue specific effects of miRNAs in different tumor types.[194]

MICRORNAS AS BIOMARKERS

The tissue-specificity of miRNA effects, combined with the association of expression changes in miRNA with human cancers of various histiotypes, has contributed to the explosion of research into the potential use of miRNAs as diagnostic and prognostic biomarkers. The Lu study was one of the earliest high-throughput screens of miRNA expression in a large group of tumors. They measured the expression of over 200 miRNAs and found that miRNA expression grouped tumors by “developmental origin”, separated normal tissues from tumor tissues, and classified poorly differentiated tumors.[175] In fact they found that miRNA classified poorly differentiated tumors better than mRNA profiling.[175] Another group developed a 48-miRNA signature that outperformed gene expression for predicting the tissue of origin in metastatic tumors.[195] Renwick and group optimized a miRNA fluorescent in situ hybridization (FISH) technique to visualize miRNA expression changes between two histologically similar skin

tumors of different cellular origin, basal cell carcinoma and Merkel cell carcinoma.[196] In addition to differentiating tumor histiotypes and distinguishing tumors from normal tissue, miRNA expression profiles can also be used to identify subtypes of a particular cancer. For example, in human breast cancer miRNA expression patterns correlate with estrogen receptor, progesterone receptor and HER2/neu receptor expression.[197-199] Finally, miRNA expression patterns can also differentiate between the so called "intrinsic" breast cancer subtypes originally defined by gene expression studies.[200-203] Unique miRNA expression patterns have been identified in prostate cancer "stem cells", a population of cells within the same tumor with increased tumorigenicity.[204] Further, a report from Croce's lab showed miRNA expression signatures associated with defined cytogenetic subgroups of acute myeloid leukemia (AML) such as 11q23 translocations and trisomy 8.[205]

Most of the reports described thus far demonstrate the association of miRNA expression levels with established, clinically relevant subtypes. However these subtypes may also exhibit different chemotherapeutic sensitivities, demonstrating the potential utility of miRNA expression profiles for determining prognosis or directing therapy. A few studies have also endeavored to establish the functional relevance of the altered miRNA expression profiles by identifying the target genes of potential biomarker miRNAs using miRNA target databases.[206-209] MicroRNA targeting of the predicted genes can be validated using reporter assays with 3' UTRs of the target genes or by immunoprecipitation of labeled miRNA-mRNA.[187] Moving to *in vitro* studies, Costinean used one of the earliest miRNA engineered animal models, Eμ-mmu-miR155 transgenic mice, to show that expression of miR155 was causative in the progression from polyclonal lymphoid proliferation to frank lymphoma or leukemia.[210] The body of functional miRNA studies in cancer continues to expand and has confirmed tumor suppressor or

oncogene functions of miRNAs in specific cancers. In fact, miRNAs regulating each of the "hallmarks of cancer"[211, 212] have been elucidated, examples of which include: miR-21 and the miR-17-92 cluster drive uncontrolled proliferation[213, 214], miR-10b contributes to invasion and metastasis[215], miR-210 stimulates angiogenesis[216], and miR-21 inhibits apoptosis.[185, 187] These functional analyses are shifting the focus on miRNAs in cancer from biomarkers to potential therapeutic targets.

MICRORNA EXPRESSION IN OSTEOSARCOMA

A growing body of literature exists exploring the significance of miRNA expression changes in OS, a tumor for which there are not yet well-established morphologic or molecular subtypes with distinct clinical outcomes. Several comprehensive reviews have been written to summarize the involvement of miRNAs in OS including a review by Ell and Kang examining the roles of miRNAs in development, bone metastases and primary bone pathologies.[217-221] Major finding in OS miRNA studies include suggested or experimentally demonstrated oncogenic or metastasis promoting roles for the miR-17-92 cluster[121, 222, 223], the miR-181 family[224, 225], miR-27a[225] and miR-21[122, 226] as well as tumor suppressive roles for miR-15/16 family members[225] and miR-34[223, 227, 228]. The roles of other miRNAs are less clear, such as the miR-29 family with reports of both elevated and decreased expression in osteosarcoma cell lines and tumors compared to "normal" controls for each sample type.[121, 223, 225] Two significant bodies of work have explored the association of miRNAs in OS with prognosis. Loss of miRNAs located in the 14q32 locus has been associated with poor patient outcome in both human and canine OS, the findings in human OS confirmed by multiple

groups.[229-231] In addition to confirming the oncogene and tumor suppressive roles of mir-27a and mir-16 respectively both *in vitro* and *in vivo*, Jones et al identified tumor-based signatures associated with "osteosarcomagenesis", metastasis and response to chemotherapy.[225] Several reports have included functional experiments confirming interactions between miRNAs of interest and genes previously identified as dysregulated in OS, such as loss of 14q32 miRNAs and miR-135 with upregulation of c-MYC, miR-34 with RUNX2, and miR-20a of the miR-17-92 cluster and Fas. [222, 228, 231, 232]

MICRORNA AND GENE REGULATORY NETWORKS

The integration of functional studies reveals key miRNA-gene interactions important for fine-tuned control of cellular processes promoting specific cancers. For example, progression of B-cell lymphoma occurs when tight control of proliferation in B-cells by a regulatory network involving *c-Myc*, the miR-17-92 cluster, and the transcription factor E2F1is disrupted.[233, 234] Other groups have similarly focused efforts on identification of miRNA-target gene regulatory networks driving processes such as proliferation and metastasis important for the progression of various cancers.[123, 193, 235] In an effort to apply a more systems biology approach to the study of miRNAs in cancer, identification of specific miRNAs and target genes on which to focus functional studies may best be accomplished by combining gene expression data and miRNA data for the same tumors. These efforts have been facilitated by the development of high throughput microarray platforms, RT-qPCR assays and sequencing strategies specifically for small RNAs. Bioinformatic tools developed in this area range from publicly available miRNA target databases to link differentially expressed miRNA-mRNA candidates to

sophisticated network analysis algorithms that will identify “hubs” or regulatory network drivers.[236-240] One example specific to osteosarcoma is a study by Poos et al in 2013 in which a combination of target prediction strategies and cluster analyses were applied to differentially expressed miRNAs and mRNAs from OS cells with high capacity for proliferation compared to their less aggressive counterparts.[122] They were able to identify several key miRNA-transcription factor co-regulatory networks associated with proliferation in cultured OS cells. Other OS studies have integrated miRNA and mRNA expression data to identify the potentially important gene regulatory miR-17-92 cluster in OS cells and miRNAs associated with the 14q32 locus in tumors.[223, 229] Revisiting the realm of biomarker discovery, several groups have begun to apply integrated miRNA and mRNA expression analysis to identify combined signatures that outperform those based on only miRNA or gene expression.[241, 242]

CIRCULATING MICRORNAS

Thus far the discussion has been focused on miRNA expression studies carried out using RNA extracted from tumors or cancer cells. Additional excitement surrounding the use of miRNAs as prognostic biomarkers is due in part to the stability of miRNAs and subsequent ability to measure cell-free miRNAs in blood products, fixed tissue and other body fluids including urine and disease-related effusions.[92, 243-247] Circulating miRNAs can be contained within microvesicles (exosomes), or in complex with proteins or lipoproteins.[248-251] Two similar studies published in 2011 showed the majority of circulating microRNAs are associated with Ago2.[249, 252] Groups showing exosomal miRNAs enter target cells where they can alter the target cell phenotype often in a pro-tumorigenic manner have recently

demonstrated the biological impact of circulating miRNAs, suggested by Valadi in 2007.[253, 254] In 2008, Lawrie et al showed that tumor-associated miRNAs were differentially expressed in the serum of patients compared to healthy controls in DLBCL, demonstrating circulating miRNA patterns in patients mirror those in their tumors. They also were among the first to demonstrate an association between elevated serum-derived miR-21 and patient outcome.[255] More recently Zhu et al suggested the picture is a bit more complicated. Their work showed that only around 10% of differentially expressed miRNAs in serum of patients compared to healthy controls were also differentially expressed in their tumors compared to normal tissues and *vice versa*.[256] Further, they found that some differentially expressed miRNAs had opposite expression pattern changes in tumors and serum.[256] While some differences may be expected due to different controls used for each comparison, their work suggests that only a portion of biomarker miRNAs identified in tumors may also be found in the serum.

A rapidly expanding body of work has identified potential circulating miRNA biomarkers for diagnosis and prognosis of a variety of human cancers.[247, 257-260] In OS specifically, expression changes in serum or plasma derived miR-9, miR-21, miR-34b, and miR-143 have all been associated with metastasis.[261-264] Additionally, synthetic miR-143 introduced into mesenchymal stem cells was released in exosomes, taken up by OS cells and ultimately contributed to reduced migration of the target OS cells.[263] Detection of miRNAs in blood components is not without its challenges and a growing body of literature has begun to address inconsistencies between reports in some of the most well studied cancers.[265, 266] Sources of variability contributing to this discord include pre-analysis factors, such as the sample-type, sample handling, blood cell contamination and hemolysis[244, 267-269] as well as platform or data analysis aspects including methods of normalization which have also plagued tissue-based

miRNA expression studies.[244, 270-272] Despite these challenges, the advantages of non-invasive circulating miRNA biomarkers in cancer remains a worthwhile pursuit as exemplified by a recent report by Kachakova demonstrating that a plasma-based miRNA signature used in combination with serum prostate specific antigen (PSA) provided increased sensitivity and specificity compared to the PSA test alone.[273]

MICRORNAS AND CHEMOSENSITIVITY

The clinical value of utilizing miRNA expression in prognosis only increases should the association between miRNA expression and outcome indicate how a patient might be expected to respond to a specific therapy. Several early reports tied miRNA expression to cell growth and apoptosis, cell processes known to contribute to responses to cytotoxic and targeted chemotherapy. For example, reports by Cheng and Chan in 2005 linked elevated miR-21 to inhibition of apoptosis in cervical and lung cancer cells.[185, 194] In 2007, Si and colleagues reported that high miR-21 expression in breast cancer cells contributed to resistance to the growth inhibitory effects of topotecan.[274] A group studying miRNA in cholangiocarcinoma was among the first to manipulate miRNA expression in cells for the purpose of examining changes in response to cancer drugs. They demonstrated that inhibition of miR-21 and miR-200b increased the sensitivity of cholangiocarcinoma cells to gemcitabine.[275] In the next few years others would establish associations between miRNA and sensitivity to targeted therapies like interferon alpha in hepatocellular carcinoma, as well as to cytotoxic doxorubicin in HCC and lung cancer, and platinum-based therapies in ovarian cancer.[276-279] Boyerinas has recently reviewed the individual miRNAs associated with cellular mechanisms of drug resistance. In

addition, an online database, pharmaco-mir, was recently established linking miRNAs with gene targets and drug sensitivity profiles.[280, 281] More recently, groups have used miRNA expression profiles to identify predictive signatures of chemoresistance. For example, a 2013 study by Croce's lab identified a 23-miRNA chemoresistance signature in ovarian cancer including evidence that the resistant cells had elevated angiogenesis.[282] A 2012 study integrated miRNA and gene expression profiles in drug-resistant breast cancer sublines to identify regulatory networks associated with tamoxifen and fulvestrant resistance.[283] Chen reported a link between circulating miRNAs and drug resistance in 2014, using a co-culture system to show miRNA-containing exosomes from docetaxel-resistant MCF-7 cells could induce a resistant phenotype in the parent MCF-7 cells.[284]

Early work exploring miRNA expression and resistance to therapy in OS came out of the Ju lab at Stony Brook University identifying roles for miR-140, via the target histone deacetylase 4 (HDAC4), and miR-215 via the target DTL in resistance of OS and colon cancer cells to methotrexate and 5-fluorouracil or tomudex respectively.[285, 286] These reports addressed two important concepts in chemoresistance: first, the effect of these resistance associated miRNAs was different in cells depending on p53 mutational status, stressing the need for genetic characterization of cancer cell models; second, chemoresistance was associated with a less-proliferative phenotype. This underscores that the role of miRNAs in cancer is complex and must be interpreted depending not only on the cancer type, but also the cellular process under examination. Other studies have established roles for miR-138 in cisplatin resistance, miR-34c in doxorubicin, cisplatin and MTX resistance, and miR-199a and miR-34a in regulation of apoptosis.[227, 287, 288] Gougelet identified miRNA signatures predictive of response to

isosfamide treatment and two groups illuminated miRNA expression changes associated with response to *pre-operative* (neoadjuvant) chemotherapy.[225, 289, 290]

Explorations utilizing genomic data to predict patient response to therapy have expanded in the era of "precision medicine" and have progressed from functional studies in individual cancers to sophisticated mathematical modeling to generate algorithms predicting sensitivity to a wide array of therapies irrespective of tumor histiotype. A collaborative study between the National Cancer Institute (NCI) and the Dialogue on Reverse Engineering Assessment and Methods (DREAM) reported by Costello et al undertook the task of evaluating 44 "drug sensitivity prediction algorithms," incorporating genomic data including both gene and miRNA expression.[291] Gene expression data was the most consistently informative among the approaches evaluated. Important to note however, is that all the top-performing models utilized multiple types of genomic data suggesting that models embracing a broader "systems biology" approach were generally more successful [291] Many of these algorithms utilized drug sensitivity data from the NCI-60 human cancer cell panel. The Developmental Therapeutics Program (NCI-DTP) maintains robust drug sensitivity data for over 40,000 compounds in this panel of human cancer cell lines (http://dtp.nci.nih.gov). Coexpression extrapolation or "COXEN" is one example of a predictive algorithm developed in the Theodorescu laboratory utilizing gene expression patterns associated with NCI-60 drug response data to predict response of independent cells or tumors to a given therapeutic."[292, 293] Since its introduction this approach has been shown to successfully predict response in bladder cancer cell lines and retrospectively predict patient response in breast cancer and ovarian cancers.[292, 294, 295] Similarly, at least four groups have undertaken high throughput miRNA expression studies in cells from the NCI-60 panel, two groups specifically addressing the association of miRNAs and

resistance to various compounds.[296-301] In Chapter Four we utilize miRNA expression and COXEN-like analyses in an attempt to predict sensitivity of OS tumors to doxorubicin.

Project Rationale

OS represents one of the most common and aggressive malignancies affecting canine patients. While rare in humans, the disease is particularly devastating because it most commonly affects children and adolescents. In both cases, standard of care treatment, even when successful is not without considerable financial, physical and emotional costs. Despite improvement in outcomes of the disease in both species with the inclusion of doxorubicin and platinum-drug based chemotherapy, survival rates have been stagnant over the past 20-30 years.[1, 34, 38] Several factors contribute to difficulty identifying novel targets and conducting clinical trials for OS. The disease is rare enough in humans to make accrual of large numbers of cases for clinical trials a difficult and lengthy process.[302] In both humans and canines, the disease is characterized by high genomic instability encompassing a complex karyotype with heterogeneous and inconsistent chromosomal aberrations.[50, 52] Thus a single pathway driving progression of OS remains elusive and is unlikely. Few clinical biomarkers exist to help stratify patients into groups that might most benefit from conventional chemotherapy or novel targeted or immunotherapy agents. The overall goal of the studies described herein was to identify clinically relevant molecular biomarkers for patient prognosis and treatment planning for canine OS. Underlying hypotheses were that aberrant gene and miRNA expression changes are associated with patient outcome and chemotherapy resistance, and implicate pathways critical to progression of OS, which could be targeted by novel treatment strategies.

Previous studies in our laboratory utilized high-throughput microarray-based gene expression profiling to explore gene expression changes between poor and good responders based on disease free interval (DFI).[91] Results of this study identified an eight gene signature that correctly classified patient into poor and good response groups. Additionally, pathway analysis based on gene expression changes showed enrichment of genes in pathways associated with bone development including Wnt/β-catenin (Wnt) and hedgehog (Hh). Despite promising results of these analyses, attempts at immunohistochemical (IHC) staining for the most deregulated genes were met with technical inconsistencies. Wnt and Hh, along with Notch, TGFβ/BMP, and fibroblast growth factor (FGF), comprise five developmental pathways contributing to normal bone development. All five of these signaling pathways have been implicated in a variety of cancers, and pharmacologic inhibitors of these pathways are in various stages of development or testing.[95]

There is considerable crosstalk between Wnt, Hh and Notch and all three pathways are considered part of a network responsible for maintenance of a stem cell phenotype.[96] In **Chapter 2 (HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors)** we elected to take a pathway-focused approach to exploration of the Notch pathway, which has been implicated in OS proliferation and metastasis in the human literature.[114, 115, 117, 122, 303] Our hypothesis was that elevations of expression of Notch pathway genes, indicative of pathway activation, would correlate with poor patient outcome. We demonstrated that microarray data from tumors and normal bone for Notch pathway related genes correctly grouped tumor and normal bone samples, but did not separate tumors into DFI cohorts. We then utilized RT-qPCR to measure expression of *NOTCH1, NOTCH2, HES1* and hair/enhancer of split related with YRPW Motif 1 (*HEY1*) in normal bone

and tumors from cohorts of dogs with DFI<100 days and DFI>300 days. We confirmed up-regulation of these genes in all tumors compared to normal bone and showed decreased expression of HES1 in tumors from dogs with DFI<100 days compared to dogs with DFI>300 days. We further confirmed an association between *decreased* expression of HES1 and poor outcome identified in our DFI cohorts using IHC in a larger representative group of canine tumors.

In order to explain the general up-regulation of the Notch pathway including increased expression of HES1 in OS tumors relative to normal bone, but decreased HES1 expression in the most aggressive tumors, we considered post-translational modifications. One recent OS paper suggested HES1 was part of an essential miRNA-mRNA regulatory hub participating specifically in proliferation of OS with regulation of HES1 by miR-9.[122] A second paper showed miR-9 was elevated in OS tumors.[121] Additionally, functional work in our laboratory confirmed oncogenic effects of insulin-like growth factor two RNA binding protein 1 (IGF2BP1), one of the eight dysregulated genes in aggressive OS showing progressively high expression from normal bone to tumors from good responders to tumors from poor responders. This protein and other dysregulated proteins in our study are part of another miRNA-gene regulatory network including let-7b and MYC.[123] We hypothesized that dysregulation of miRNAs were likely contributing to the gene or protein expression changes associated with an aggressive OS phenotype and may themselves provide excellent biomarkers for prognosis.

MiRNAs are small non-coding RNAs involved in regulation of gene expression that are also involved with processes contributing to cancer.[167] MiRNAs are attractive biomarkers with increased stability in biological fluids and fixed tissues.[243, 304] These features are ideal for the veterinary setting where the cost and stringent requirements for collection and storage of

pristine samples for mRNA or protein assays are not always practical. In **Chapter 3 (Tumor and serum-derived miRNA expression changes associated with poor outcome in canine osteosarcoma)** we utilized RT-qPCR for miRNA expression profiling in normal bone and tumors from our DFI cohorts. Our main goal was to identify predictive miRNA signatures associated with patient outcome in OS tumors and in serum. An underlying objective of these studies was to develop predictive prognostic screens using miRNA for OS where histologic subtypes and gene expression classifications have thus far failed to show consistent clinical relevance. We identified 20 candidate miRNAs to measure in 33 additional tumors and 32 serum samples from canine patients. Additionally we used miRNA-mRNA interaction analyses to predict potential specific interactions or pathways contributing to OS. We identified a 3-miR signature and a 2-miR signature in tumors and serum samples respectively, which successfully separated patients into two distinct outcome groups. Additionally, we found potential evidence for miRNA dysregulation contributing to Notch activation in tumors compared to normal bone and to the up regulation of IGF2BP1 in aggressive tumors. We not only identified miRNA signatures associated with outcome, but our miRNA-mRNA interaction analyses identified potential activation of pathways targeted by currently available small molecule inhibitors.

Molecular biomarkers of prognosis have even more value if they prove to identify targetable pathways themselves or provide a means to direct treatment. Several host and tumor factors can contribute to progression of cancer to metastasis despite treatment including resistance to chemotherapy. MiRNAs may contribute to a resistant phenotype by inhibiting or enhancing cellular processes such as apoptosis or angiogenesis or by direct regulation of drug targets or other proteins involved in drug resistance.[305] Given the complex factors at play within spontaneous tumors, cell models with experimentally established thresholds for drug

sensitivity are needed to tease out the association of miRNA and gene expression and resistance. Complex predictive modeling techniques have been embraced to utilize cell models and genomic tools to approach the problem of identifying subpopulations of patients most likely to benefit from a specific therapy. One such algorithm is the co-expression extrapolation (COXEN) method, which Jared Fowles in the Gustafson laboratory at the Colorado State University Flint Animal Cancer Center recently adapted for use in canine OS. His work produced predictive models based on gene expression in human or canine OS cells to predict sensitivity of OS tumors to doxorubicin or carboplatin, the two drugs used most commonly in the treatment of canine OS.

In **Chapter 4 (Utilization of miRNA expression changes in OS cancer cells associated with drug sensitivity to identify drug-associated miNRAs associated with outcome in canine tumors)** we used the Affymetrix Genechip miRNA4.0 micoarray to profile miRNA expression in 29 canine cancer cell lines for which we also had drug sensitivity data for common cytotoxic chemotherapies. Our goals here were to identify miRNA expression changes associated with resistance to doxorubicin and/or carboplatin, the two most commonly used chemotherapies for canine OS. Our hypothesis was that miRNA expression changes could be used in predictive modeling schemes, such as COXEN, to predict patient outcome for patients whose treatments included these drugs. We utilized COXEN-type analyses to identify candidate drug-resistance-associated miRNAs to measure and evaluate in the 33 canine OS tumors in our patient set (COS33). We identified miR-98 as an additional predictive miRNA participating in a 4-miR signature associated with patient outcome. We developed a modified COXEN analysis to predict sensitivity of 23/33 of the COS33 tumors to doxorubicin. While our results did not reach significance, we remain encouraged that predictive modeling using miRNAs will be successful or inclusion of miRNA expression into methods like COXEN will improve the accuracy of these

predictive models. MiRNA-mRNA interaction analyses between dysregulated miRNAs in this study and genes from Jared Fowles' successful COXEN models suggest a role for Notch signaling in doxorubicin resistance. Finally, pathway analysis of the top 5 miRNAs associated with outcome in our work suggests enrichment of the PI3K-AKT pathway, components of which are also targeted by currently available drugs.

The overall goal of this body of work was to identify molecular biomarkers of progressive disease in OS. Additionally, gene and miRNA expression changes associated with outcome were used to identify pathways contributing to aggressive OS and resistance to chemotherapy. Validation of our predictive models as well as targetable pathways identified by miRNA-gene interaction analyses may provide useful biomarkers to stratify patients likely to benefit from novel therapies in OS.

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Chapter 2

HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors.

SUMMARY

Hairy and enhancer of split 1 (HES1), a basic helix-loop-helix transcriptional repressor, is a downstream target of Notch signaling. Notch signaling and HES1 expression have been linked to growth and survival in a variety of human cancer types and have been associated with increased metastasis and invasiveness in human osteosarcoma cell lines. Osteosarcoma (OSA) is an aggressive cancer demonstrating both high metastatic rate and chemotherapeutic resistance. The current study examined expression of Notch signaling mediators in primary canine OSA tumors and canine and human osteosarcoma cell lines to assess their role in OSA development and progression.

Reverse transcriptase - quantitative PCR (RT-qPCR) was utilized to quantify *HES1, HEY1, NOTCH1 and NOTCH2* gene expression in matched tumor and normal metaphyseal bone samples taken from dogs treated for appendicular OSA at the Colorado State University Veterinary Teaching Hospital. Gene expression was also assessed in tumors from dogs with a disease free interval (DFI) of <100 days compared to those with a DFI>300 days following treatment with surgical amputation followed by standard chemotherapy. Immunohistochemistry was performed to confirm expression of HES1. Data from RT-qPCR and immunohistochemical (IHC) experiments were analyzed using REST2009 software and survival analysis based on IHC

expression employed the Kaplan-Meier method and log rank analysis. Unbiased clustered images were generated from gene array analysis data for Notch/HES1 associated genes.

Gene array analysis of Notch/HES1 associated genes suggested alterations in the Notch signaling pathway may contribute to the development of canine OSA. *HES1* mRNA expression was elevated in tumor samples relative to normal bone, but decreased in tumor samples from dogs with a DFI < 100 days relative to those with a DFI > 300 days. *NOTCH2 and HEY1* mRNA expression was also elevated in tumors relative to normal bone, but was not differentially expressed between the DFI tumor groups. Survival analysis confirmed an association between decreased HES1 immunosignal and shorter DFI.

Our findings suggest that activation of Notch signaling occurs and may contribute to the development of canine OSA. However, association of low HES1 expression and shorter DFI suggests that mechanisms that do not alter HES1 expression may drive the most aggressive tumors.

INTRODUCTION

Osteosarcoma (OSA) is the most common malignant bone tumor among children and adolescents with an incidence of 4.4 cases per million per year in the United States [1]. OSA is also the most common spontaneous primary bone tumor of dogs, estimated to affect greater than 8,000 dogs annually in the United States [2]. Tumor morphology, biological behavior, progression of disease and molecular characteristics are very similar in dogs and humans [2-7]. Consequently, dogs provide a valuable comparative model of human OSA. Standard of care therapy for both human and canine OSA patients remains a combination of surgery and chemotherapy, with five-year survival rates reported in humans as high as 70% [1, 8] and median

survival in canine patients around 200 days [2]. Unfortunately, in both human and canine patients approximately 80% are estimated to have micrometastases at presentation, some of whose tumors are also refractory to chemotherapy [2, 8]. These patients continue to have a poor prognosis. Histologic classification alone has not proven clinically relevant for determination of tumors likely to metastasize or exhibit resistance to chemotherapy protocols. The focus of recent research, therefore, has turned toward molecular characterization of primary tumors, especially aberrant gene and/or protein expression that might correlate with prognosis or chemotherapy sensitivity.

Hairy and enhancer of split 1 (HES1), a basic helix-loop-helix (bHLH) transcriptional repressor, is a downstream target of the Notch signaling pathway. The intracellular domain of activated Notch receptors (NICD) translocates to the nucleus, forms a transcriptional activating complex with recombination signal binding protein for immunoglobulin kappa J region (RPBJκ) and activates expression of target genes including HES1 [9, 10]. The HES1 protein contains both DNA-binding and protein-protein interaction domains important for its function as a transcriptional regulator (including negative regulation of its own transcription) [9, 11, 12]. Notch-independent HES1 expression can also result from Hedgehog and c-Jun N-terminal kinase (JNK) signaling as well as from RAS/MAPK signaling [10, 13-15]. Regulation of HES1 expression and activity is dependent on the tissue, spatial and temporal factors, and the proteins with which it interacts [9, 10].

Overexpression of Notch and/or HES1 is associated with a variety of human cancers including T-cell acute lymphoblastic leukemia (ALL), and ovarian, breast, cervical, prostate, colon and non-small cell lung cancers [16-19]. Notch /HES1 has also been shown to have tumor suppressor activity in some cancers including hepatocellular carcinoma, B-cell ALL, myeloid

leukemia and neuroblastoma [20-23]. In human OSA, Notch is implicated in OSA cell proliferation, invasion and metastasis [24, 25]. Increased HES1 mRNA expression was shown in some human OSA cells and OSA tumor samples compared to osteoblasts or normal bone and an association between high HES1 expression and decreased survival of OSA patients has been suggested [24-27]. Reduced invasiveness in response to suppression of Notch signaling and HES1 activity implicates Notch/HES1 signaling in metastasis [28]. Another study suggests both up-regulation of Notch and increased expression of HES1 in one OSA cell line occurs in response to activation of the Wnt/β-catenin pathway [29].

During bone development there is significant cross talk between the Wnt/β-catenin, hedgehog, and Notch pathways affecting osteoblast differentiation and maturation and influencing HES1 expression [10, 29-31]. Like Notch and Wnt/β-catenin, aberrant hedgehog signaling is also associated with development of human cancers [31]. Previous studies in our lab identified decreased expression of three hedgehog pathway associated genes in OSA tumors from dogs with a disease free interval (DFI) < 100 days (poor-responders) compared with tumors from dogs with a DFI>300 (good-responders) [32].

In order to explore the hypothesis that Notch signaling would be altered in canine OSA compared to normal bone samples, the current study examines the expression of NOTCH1 and 2 receptors and signaling targets, HES1 and HEY1, in canine OSA samples from patients with known outcome and normal bone tissues. Immunohistochemical analysis of HES1 protein was assessed in Kaplan-Meyer survival analysis to confirm the association of decreased HES1 expression with a shorter DFI.

METHODS

Tumor Donors

Chemotherapy-naïve primary tumor samples were selected from the Colorado State University (CSU) Flint Animal Cancer Center's tissue archive. Samples are archived with owner consent and approval by the CSU Institutional Animal Care and Use Committee. Twenty tumors from good- and poor-responders (n=10 each group) were selected following the protocol previously published [32]. Briefly, chemotherapy-naïve primary OSA samples were from dogs treated with surgical amputation followed by chemotherapy with doxorubicin and/or a platinum based drug (distribution of choice of drug was not significantly different between groups). All twenty dogs were free of thoracic metastases by radiographic analysis at diagnosis and follow up consisted of evaluation by clinical examinations including thoracic radiographs every 2-3 months after initial treatment. Disease free interval (DFI) was calculated from surgery until development of metastatic disease and samples were identified for cohorts of good responders (DFI>300 days) and poor responders (DFI<100 days) in order to flank the median DFI (200 days). Nine additional appendicular OSA tumor samples were collected from which matched normal metaphyseal bone was harvested from the same limb (at least one joint space away from the tumor) following amputation. These nine matched samples were collected at amputation as cases came in (convenience sample) and absence/presence of metastasis, post-operative treatment, and patient follow-up were less consistent in this population. Tumor and normal bone fragments collected at amputation were flash-frozen in liquid nitrogen and stored at -80^{o} C. Tumor fragments were also fixed in 10% neutral buffered formalin for 24 hours with subsequent routine processing and paraffin embedding.

Immunohistochemical HES1 expression was also assessed in a subset of canine appendicular OSA patients from a previously reported multi-institutional randomized prospective clinical trial [33]. The study was approved by the Institutional Animal Care and Use Committees of the participating institutions. All dogs underwent amputation followed by 5 cycles of adjuvant doxorubicin, with or without an investigational matrix metalloprotease inhibitor. Inclusion/exclusion criteria, staging, and follow-up procedures were standardized and tumor tissues were processed as previously reported [33]. Histologic grading (from 1 to 3) was performed by one author (BEP) utilizing a schema incorporating amount of matrix, percent necrosis, nuclear pleomorphism, nucleolar size/number and mitosis score [33]. Mitotic index was calculated by counting the number of mitotic figures per 10 random 400X fields.

Cell Culture

Canine cell lines used in this study were provided by Dr. Douglas Thamm; all cell lines were validated for species and genetic identity using short-tandem-repeat (STR) profiling as previously described [34]. Human OSA cell lines were obtained from Dr. Douglas Thamm (MG63, SAOS-2, SJSA-1), Dr. Hue Luu (MG63.2), or purchased from ATCC (U2OS). The MG63.2 cell line is a metastatic sub-line of the MG63 line, obtained via serial passage of rare lung metastases from MG63 [35]. All non-purchased cell lines were validated prior to use using STR profiling by the University of Colorado DNA Sequencing Shared Resource. Cells were cultured in C10 media (DMEM high glucose with 4 mM L-glutamine (Hyclone Laboratories, Inc.), 1mM of sodium pyruvate, 2x MEM vitamins, 1x MEM non-essential amino acids, 1x antibiotic-antimycotic (100x: 10,000 IU/ml penicillin, 10,000 ug/ul streptomycin and 25ug/ml)

(all additives from Mediatech, Inc.), and 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO).

RNA Extraction

Total RNA was extracted from tumors and RT-qPCR was conducted as described previously [32]. Briefly, samples were freeze-fractured, homogenized, extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy clean up (Quiagen, Valencia, CA) following manufacturer's protocols. RNA was extracted from normal bone using the same protocol with an additional spin of 800 x g at 4^{o}C for 5 minutes following homogenization. The supernatant was carried forward through the Trizol protocol. Total RNA was extracted from human and canine OSA cells using the RNeasy Kit (Qiagen) per the manufacturer's protocol. RNA was quantified via spectrophotometry and bioanalyzed for integrity as described in O'Donaghue et al [32] with samples used having a RNA integrity number of at least 8. Human adult osteoblast total RNA was purchased from CELL Applications, Inc.

Reverse transcriptase PCR and quantitative real time PCR

cDNA synthesis was completed using the QuantiTect Reverse Transcription Kit (Qiagen) with 1 or 3 μg input RNA. RT-qPCR of cDNA was run using iQ SYBR Green Supermix (Bio-Rad) and 25 ng equivalent RNA input in 25 μL reactions on a Stratagene Mx3000P instrument. Expression in canine cells and tissues was normalized to hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) expression. *HPRT1* was selected based on its consistent moderate expression in our sample sets in prior microarray and RT-qPCR analysis [32] and its previous use as a canine reference gene [36]. Consistent with current recommendations for the

selection of reference genes and because no single reference gene exhibited unchanged expression between samples, expression in human OSA cells was normalized to the geometric mean of four reference genes; ribosomal protein S15 (*RBS15*), glyceraldehyde-3-dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18SrRNA*) and *HPRT1* [37]. Primer sequences and efficiencies for all genes and the full sequence of the canine *HES1* amplicon are listed in (Table 2.1). Primers were designed using Primer-Blast based upon NCBI RefSeq mRNA sequences when available. Primers were designed to be intron spanning when possible and cross-checked for specificity via UCSC in silico PCR. Primers were further validated with standard curves to calculate efficiency, and dissociation curves as previously described [34]. RT-qPCR products were validated for size by agarose gel electrophoresis and sequenced to confirm identity. The 161 bp canine *HES1* amplicon revealed 98% homology to the human homolog of *HES1*. Human *HES1* primers used were the same as those used by Zhang et al. [24]. The identity of the 200 bp amplicon was verified as human *HES1* by dideoxy sequencing (CSU DNA sequencing Core).

Table 2.1: Sequences, amplicon sizes, and efficiencies of primer pairs used in RT-qPCR experiments.

Gene		Primer Sequence (5' to 3')	Amplicon	Avg Primer Eff. (%)
HPRT1	F	TGC TCG AGA TGT GAT GAA GG	192	90
	R	TCC CCT GTT GAC TGG TCA TT		
RPS15	F	TTC CGC AAG TTC ACC TAC C	361	95
	R	CGG GCC GGC CAT GCT TTA CG		
GAPDH	F	ACC ACA GTC CAT GCC ATC AC	268	95
	R	CCT GCT TCA CCA CCT TCT TGA		
18SrRNA	F	GAG GCC CTG TAA TTG GAA TGA G	120	95
	R	GCA GCA ACT TTA ATA TAC GCT ATT GG		
HES1 (ca)	F	CAT CCA AGC CTA TCA TGG AGA	161	105
	R	GTT CCG GAG GTG CTT CAC T		
HES1 (hu)	F	ACG ACA CCG GAT AAA CCA AA	200	105
	R	CGG AGG TGC ACT GTC AT		
NOTCH1	F	CAT CAT CAA TGG CTG CAA GGG	126	81
	R	TCA TTC TCA CAC GTG GCA CC		
NOTCH2	F	TCG GGA TAG CTA TGA GCC CT	188	99

	R	GGC ATG TTG CTT TCC CCA AC		
HEY1	F	ACC TGA AAA TGC TGC ACA CG	195	89
	R	GCT GGG AGG CGT AGT TGT TA		

Hes1 Amplicon sequenced contiguous product :
CATCCAAGCCTATCATGGAGAAAAGACGAAGAGCAAGGATAAATGAAAGTCTGAG
CCAGCTGAAAACACTGATTTTGGATGCTCTTAAGAAAGATAGCTCGCGGCATTCCA
AGCTGGAGAAGGCGGACATTCTGGAAATGACAGTGAAGCACCTCCGGAAC

Western Blot

Western blot analysis was performed on canine and human OSA cells using whole cell lysates or cytoplasmic and nuclear fractions. Whole cell lysates were prepared in triethanolamine (TEA) lysis buffer (55 mM TEA, pH 7.5, 111 mM NaCl, and 2.2 mM EDTA, 0.44% SDS) with 1X Complete Protease Inhibitor Cocktail (Roche Diagnostics). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific). Nuclear extracts were prepared using a hypotonic 0.5% or 0.25% IgePal (NP-40) buffer (10 mM Hepes, 1.5 mM MgCl, and 10 mM KCl). Briefly, harvested cell pellets were re-suspended in IgePal buffer with protease inhibitor while vortexing, incubated on ice for 0-5 minutes, and centrifuged for 5 minutes at 500 x g. The supernatant (cytoplasmic fraction) was collected and the pellet (nuclear fraction) was re-suspended in TEA lysis buffer with protease inhibitors. Samples were separated using SDS-PAGE and transferred to a polyvinylidine fluoride membrane. The membrane was blocked with 5% non-fat dry milk (NFDM) for one hour at room temperature and incubated with rabbit monoclonal anti-HES1 antibody (RabMAb EPR4226, 1:500; Epitomics) in 5% bovine serum albumin (BSA) at 4º C overnight. After washing in 0.1% Tween 20-Tris-buffered saline (TBST) the membrane was incubated with secondary horseradish peroxidase conjugated goat anti-rabbit antibody (1:5000; Bio-Rad) in 5% NFDM for one hour at room temperature. SuperSignal West Dura Extended Duration Substrate

(Pierce Biotechnology) was used to detect chemiluminescent signals. Band intensity from four experiments using whole cell lysates from MG63 and MG63.2 cell lines were analyzed using ImageJ software. The intensity of the HES1 band was normalized to the corresponding α-tubulin loading control.

Immunohistochemistry (IHC)

IHC to detect HES1 expression was performed on 4 μm sections from formalin-fixed paraffin embedded (FFPE) tumor tissues using standard immunoperoxidase techniques on charged slides with hematoxylin counter stain. Slides with sections were heated at 60^{o}C for 30 minutes, allowed to cool, and deparaffinized with xylene or a citrus based clearing solution (Thermo-Fisher Scientific), and rehydrated with descending ethanol concentrations in deionized water (100%, 95%, 75% and 50%). Heat induced epitope retrieval was done with 10 mM sodium citrate buffer (pH 6.0) heated in a pressure cooker for 1 minute at 125^{o} C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 5 minutes with 3 washes in TBST both before and after. Slides were incubated with a non-serum protein block (Background Sniper, Biocare Medical) at room temperature for 15 minutes followed by incubation with primary antibody overnight at 4^{o}C overnight. The primary antibody (anti-HES1 RabMAb, Epitomics) was used at a dilution of 1:750 (diluted in Antibody Diluent, Dako). Sections were then incubated with a prediluted secondary antibody conjugated to horseradish peroxidase (Envision and Dual Link System HRP, Dako) for 30 minutes at room temperature with 3 TBST washes both before and after. Diaminobenzidine (DAB, Ventana Medical Systems) was used as a chromogen for immunoreactive complex detection and slides were counterstained with hematoxylin.

Sixty-one additional FFPE tumor samples were analyzed for HES1 immunohistochemical expression utilizing a protocol similar to that described above with the following exceptions: primary antibody was diluted in 2.5% normal goat serum in TBST (1:750 or 1:375, higher antibody concentration was used in subsequent batches to increase immunoreactivity signal), and detection was performed using biotinylated anti-rabbit IgG antibody in a Vectastain ABC Kit (Vector Laboratories). The IHC was performed in five batches of 8 to 18 slides each with the same antibody dilution used for an entire batch. Variations in antibody dilutions were controlled for by inclusion of a positive control tumor slide with a total immunoreactivity score of 4 (percent cells staining score of 2 and intensity score of 2; Table1). All samples within each batch were scored in reference to the control. Negative controls lacking primary antibody were included in each batch.

HES1 antibody validation was done using human placenta and canine lung and pancreas as positive control tissues. Specificity of the primary antibody was verified using a HES1 blocking peptide (Epitomics). Briefly, primary antibody was incubated with 25x (by mass) blocking peptide in antibody diluents (at both 1:375 and 1:750) for one hour at room temperature before application to canine control and sample tumor slides. Positive and negative controls with sections from the same tissues were incubated in parallel.

Immunohistochemical scoring of all slides was performed independently by two authors blinded to case information. A positive cell was any neoplastic cell with distinct brown staining in the nucleus (stromal cells and endothelial cells were not counted). The percentage of positive cells in each sample was estimated based on an average of two or more high powered fields and scored as follows, 1: < 50% cells stain positive, 2: 50-75% cells stain positive, 3: >75% cells stain positive. Average stain intensity ranged from 1 to 3 (lowest to highest intensity). Field

location and number were selected randomly at the discretion of the individual scorer. The product of the percentage and intensity scores made up the overall immunoreactivity score (ranging from 1 to 9). Both scorers simultaneously reviewed slides with conflicting scores (scores deviating by more than 1 in either category) (n=5) and consensus was reached. After review, total scores were averaged for statistical analyses.

Immunocytochemistry (ICC)

Immunocytochemistry was performed utilizing the same reagents and a similar protocol to that used for IHC. Slides were prepared via cytospin and dried overnight. Prior to the blocking step cells were fixed with 100% methanol at room temperature for 15 minutes, allowed to dry, washed in TTBS and incubated in 0.1% TritonX-100 in TBS for 7-12 minutes. The remainder of the procedure was identical to that used for IHC, but a higher concentration of primary antibody (1:250) was used.

Photomicrographs (IHC and ICC) were taken using the Olympus BX51 Research System Microscope with an Olympus dp70 Digital Camera System. Minimal additional editing was done in Microsoft ® PowerPoint ® for Mac 2011.

Gene expression microarray analysis

Total RNA from primary OSA tumor samples from dogs with DFI<100 (n=8) and DFI>300 (n=7) was analyzed on GeneChip Canine 2.0 Genome Arrays (Affymetrix, Santa Clara, CA) at CSU's Rocky Mountain Regional Center for Excellence (RMRCE) Genomics Core per Affymetrix protocols as described [35]. Normal bone samples (n=8) were analyzed using an identical protocol. Samples used for microarray analysis were a subset of those used for RT-

qPCR (microarray samples were limited due to array costs). Microarray pre-processing combining the osteosarcoma samples with the normal bone samples was conducted using Probe Logarithmic Intensity Error (PLIER) estimation algorithms with $\log_2$ transformations. Probesets including Notch receptor ligands, effectors, or targets of either the canonical Notch pathway or HES1 were selected based on literature review, Ingenuity® Systems Pathway analysis, and/or inclusion in The Human Notch Signaling Pathway RT² Profiler™ PCR Array (SAbiosciences) (Additional file 1). CIMminer was used to generate clustered images of the data from the 75 selected probesets with unsupervised clustering on both axes and the following parameters: average linkage, Euclidean distance, and quantile binning with median centering of the data. Full microarray data for the DFI groups is available through NCBI's Gene Expression Omnibus (GEO) via accession number GSE24251.

Statistics

Statistical analysis of RT-qPCR and immunohistochemistry data (not including survival data) was performed using Prism software (GraphPad Software, La Jolla, CA). For RT-qPCR data standard curves, dissociation curves and amplification data was collected on a Stratagene Mx3000P instrument and analyzed using the Rest2009 software [38]. *HES1* RT-qPCR data was also analyzed using the $2^{(-\Delta\Delta Ct)}$ method [39] with similar results. IHC scores for the DFI>300 and DFI<100 tumors were analyzed with a 2-tailed Fischer's exact test after separating scores into low expression (total score less than 4) and high expression (total score greater than or equal to 4) categories. The cut off was based on results of receiver-operating characteristic (ROC) analysis of immunohistochemical scores for the DFI>300 and DFI<100 groups. Welch t-test in ArrayTrack 3.5.0 with false discovery rate correction for multiple comparisons (FDR; based on

all array probesets) was used to compare microarray gene expression data. Significance was defined as $p<0.05$ (Welch t-test) or $q<0.05$ (FDR).

Statistical analysis of survival data was performed using a combination of Prism and SPSS software version 20 for Macintosh (IBM, Armonk, NY). Correlations between HES1 expression levels and other markers on a continuous scale were evaluated using linear regression analysis. A 2-tailed, unpaired t-test was used to evaluate the association between HES1 expression levels and categorical markers. The median DFI was estimated using the Kaplan-Meier method, and comparisons between groups made using log rank analysis for categorical variables. For continuous variables, markers were categorized into a low and high group using the median value as the break point. Multivariable Cox regression analysis was then performed, utilizing both forward and backward stepwise models. Variables identified with a univariate p-value of <0.1 were included in the multivariate analysis. For all other tests, p-values of <0.05 were considered significant.

RESULTS

Gene expression analysis of Notch/HES1-associated genes groups normal and OSA bone samples, but does not distinguish DFI groups

To assess the biological relevance of Notch/HES1 signaling in canine osteosarcoma, probesets including Notch receptor ligands, effectors, or targets of either the canonical Notch pathway or HES1 were selected from Canine 2.0 gene array data and analyzed for differential gene expression as described in materials and methods. Unbiased cluster analysis of data for the 51 Notch/HES1-associated genes separated normal bone from tumors, but did not discriminate between the DFI groups (Figure 2.1). In total, 30 of 51 (58.8%) Notch/HES1 pathway associated

genes examined were significantly different between tumor and normal bone ($p<0.05$, $q<0.05$); 23/30 (76.7%) had increased expression in tumors. Specifically, mRNA expression of *NOTCH1* and *NOTCH2* was elevated in tumor samples compared to normal bone ($p<0.05$, $q<0.05$). None of the genes evaluated had significantly different expression between DFI groups when corrected for multiple comparisons. *HES1* was not included on the Canine 2.0 chip, but *HEY1*, another Notch target, was also elevated in tumors compared to normal bone ($p<0.05$, $q<0.05$).

RT-qPCR analysis for *NOTCH1, NOTCH2, HEY1 and HES1* was conducted on the normal bone/matched OSA and DFI tumor sample sets (Figures 2.2 and 2.3). *NOTCH1* exhibited decreased expression in the DFI<100 day group relative to normal bone (FC down – 1.656, $p<0.001$), with no other significant changes measured. This result differed from the 1.27 fold upregulation of *NOTCH1* identified in the gene array analysis, however previous studies have shown that fold-change differences <1.5 are frequently unreliable [40]. Consistent with the array data, *NOTCH2* exhibited an approximate 4-fold elevation in expression in both sets of DFI tumors, separately and in combination, relative to normal bone ($p<0.001$). Similarly, *HEY1* expression was elevated in each tumor group by a fold-change ranging from 6 to 10.2 ($p\leq0.001$). RT-qPCR analysis of these Notch signaling pathway elements confirmed our finding that Notch signaling is elevated in tumors relative to normal bone, but not between tumors in the two DFI groups.

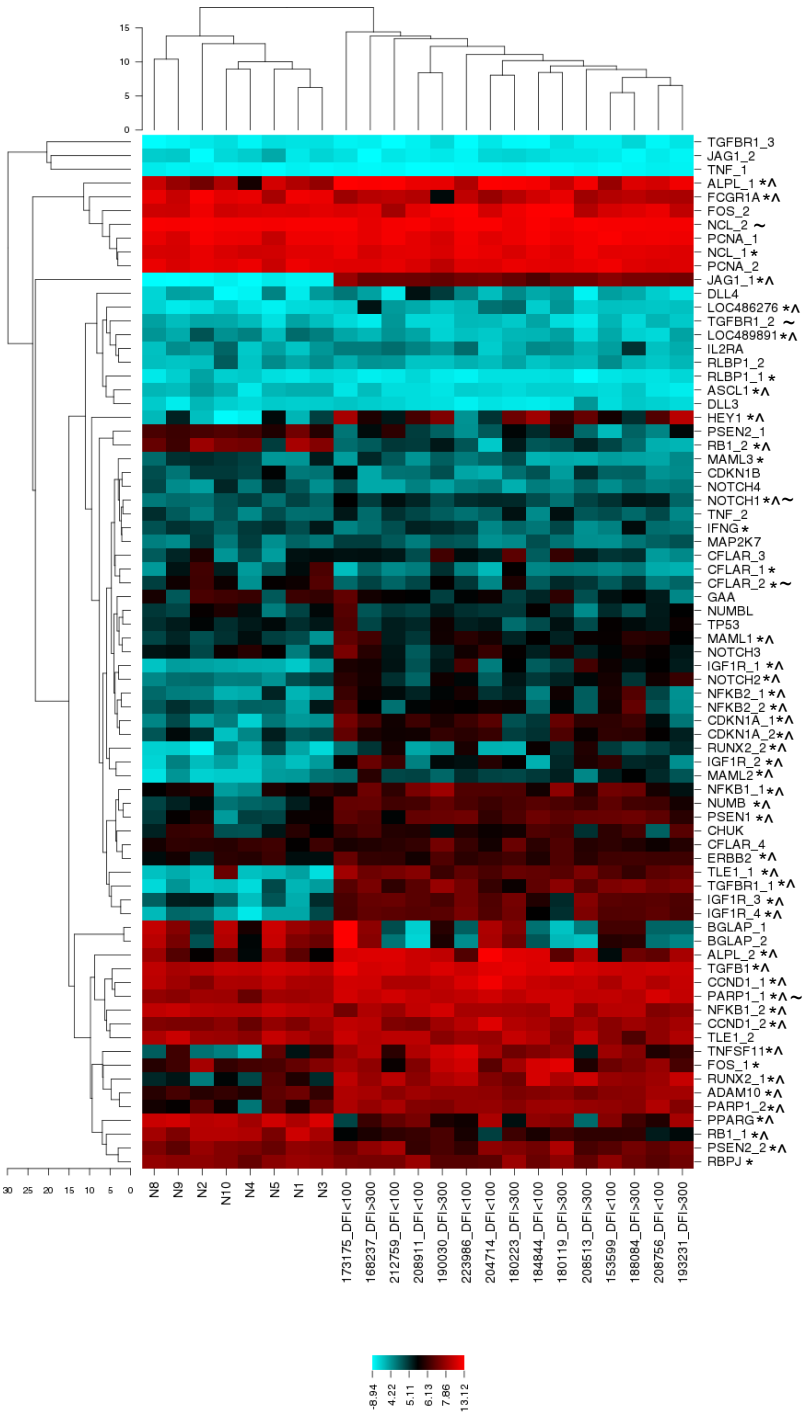


Figure 2.1 Differential expression of Notch/HES1-associated genes in canine osteosarcoma. Unbiased cluster analysis separates normal bone from tumors, but does not discriminate DFI<100 day and DFI>300 day primary tumors groups. An asterisk (*) and a caret (^) denote genes significantly different between tumor and normal bone (* $p<0.05$, ^ $q<0.05$). Genes different between DFI groups ($p<0.05$) are denoted by (~). Multiple probesets are present for some genes. LOC486276 = Deltex 1 homolog (DTX1), LOC489891 = LFNG O-fucosylpeptide

3-beta-N-acetylglucosaminyltransferase/lunatic fringe (LFNG). Colored bar below indicates the intensity scale of $\log^2$ transformed expression values.

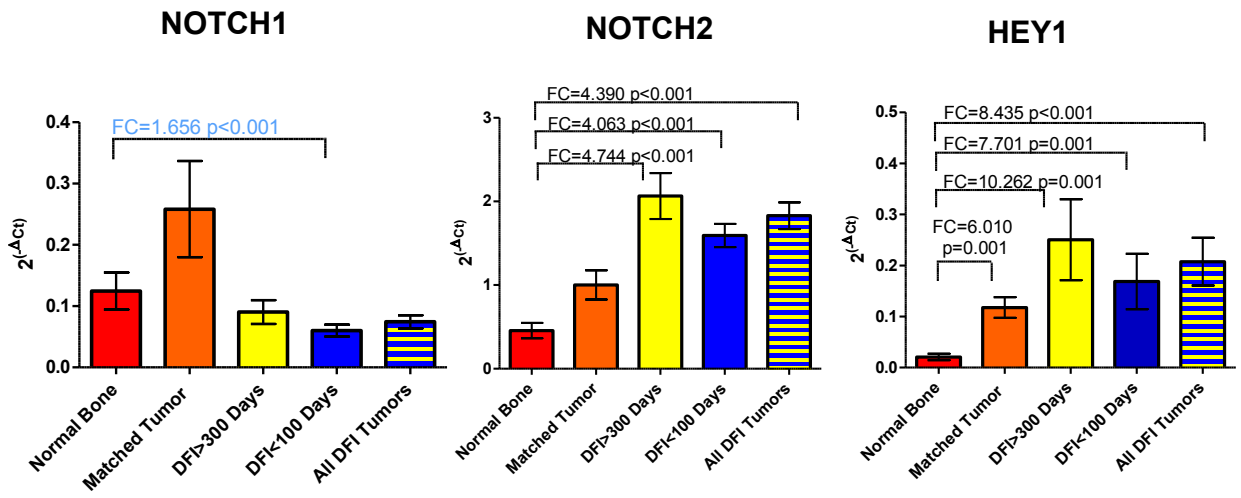


Figure 2.2. Expression of *NOTCH1*, *NOTCH2* and *HEY1* mRNA in canine normal bone and osteosarcoma (RT-qPCR). *NOTCH1*, *NOTCH2,* and *HEY1* mRNA expressed as $2^{(-\Delta CT)}$ normalized to *HPRT1* is shown for normal bone (n=9), matched tumors (n=9), tumors from dogs with DFI>300 days, tumors from dogs with DFI<100 days, and combined DFI group tumors. Comparisons of each tumor group relative to normal bone and DFI<100 relative to DFI>300 day groups were analyzed with REST 2009 software and significant fold changes are indicated by brackets on the graph. Values in blue indicate the reduced fold-change expression in DFI<100 compared to normal bone. Bars represent mean ± SEM.

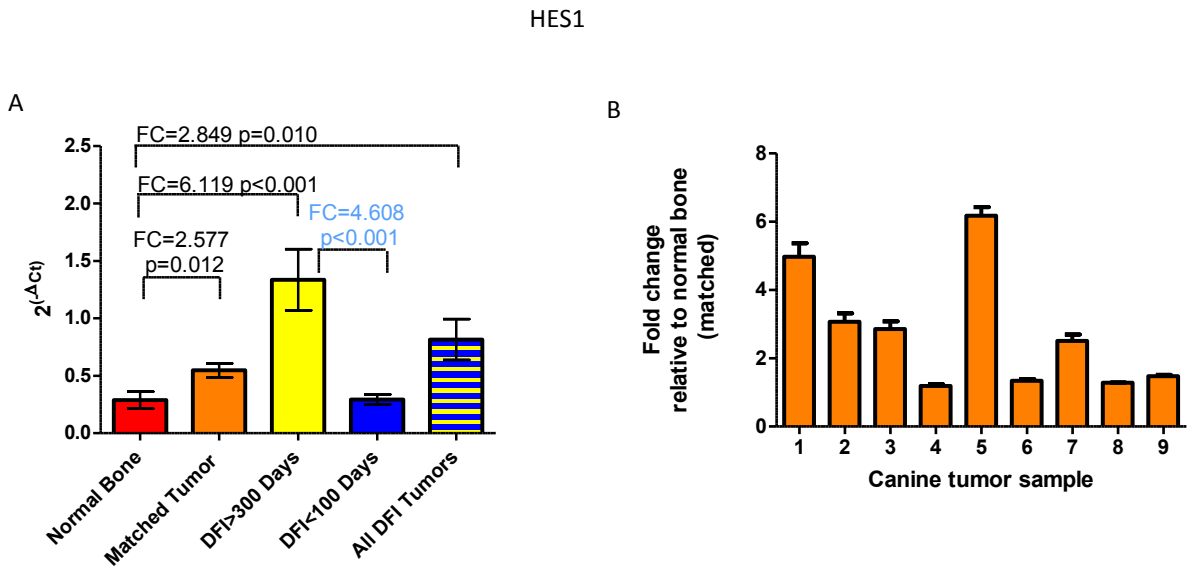


Figure 2.3. Expression of *HES1* mRNA in canine normal bone and osteosarcoma (RT-qPCR). (A) *HES1* mRNA expressed as $2^{(-\Delta CT)}$ normalized to *HPRT1* in normal bone (n=9), matched tumors (n=9), tumors from dogs with DFI>300 days (n=10), tumors from dogs with DFI<100 days (n=10), and combined DFI group tumors. Comparisons of each tumor group relative to normal bone and DFI<100 relative to DFI>300 day groups were analyzed with REST 2009 software and significant fold changes are indicated by brackets on the graph. Values in blue indicate reduced fold-change in DFI<100 relative to DFI>300 group. (B) Fold change in expression calculated using the comparative Ct ($2^{(-\Delta\Delta Ct)}$) method between each canine tumor and its matched normal bone sample (normalized to HPRT1). Bars represent mean ± SEM.

HES1 mRNA expression in tumors and its prognostic significance

RT-qPCR was also used to assess *HES1* mRNA levels in OSA tumor and matched normal bone samples. Average *HES1* mRNA expression was elevated 2.57-fold in canine OSA tumors compared to the matched normal bone (Figure 2.3A; p=0.012); however, this fold change was highly variable when each OSA tumor was compared to its matched normal bone sample, with 5 tumors exhibiting elevated expression compared to normal bone and 4 tumors having virtually unchanged expression (Figure 2.3B, range 1.19-6.17-fold).

We also assessed mRNA levels for *HES1* in tumors taken from dogs with a DFI <100 days or DFI >300 days following treatment by amputation and chemotherapy. We found that *HES1* expression was elevated 4.608-fold in the DFI>300 tumors compared to the DFI<100 group (Figure 2.3A; $p<0.001$). *HES1* expression in the DFI<100 group was not different from the normal bone samples.

Messenger RNA levels of HES1 were measured in canine and human osteosarcoma cell lines and confirmed using Western blot analysis using a rabbit monoclonal anti-human HES1 antibody as described to determine if *HES1* mRNA levels correlated to protein expression, (Figures 2.4 and 2.5, Additional File 3). Comparison of canine and human amino acid sequence of the *HES1* gene identified 86% homology in the epitope targeted by this antibody. This was based on the predicted amino acid sequence of NCBI reference sequence XM_548669.1, which has been removed as a result of standard genome annotation processing. No additional canine *HES1* record is currently available. Western blot analysis of whole cell OSA cell lysates revealed a 30 kD protein (HES1) as well as larger non-specific bands (Figure 2.4A, W). Given the role of HES1 as a transcriptional regulator, we hypothesized that active HES1 protein would reside in the nucleus. Western blot analysis of isolated nuclear and cytoplasmic fractions from both canine and human OSA cell lines confirmed enrichment of the 30 kD HES-1 protein in the nuclear fraction (Figure 2.4A, N) while the non-specific bands were enriched in the cytoplasm fraction (Figure 2.4A, C). Since equal amounts of total protein were loaded in each lane, the increased intensity and/or number of nonspecific bands in the cytoplasmic fraction were likely the result of concentration of these cytoplasmic proteins relative to total protein. Experiments using human OSA cells showed similar results (Figure 2.6).

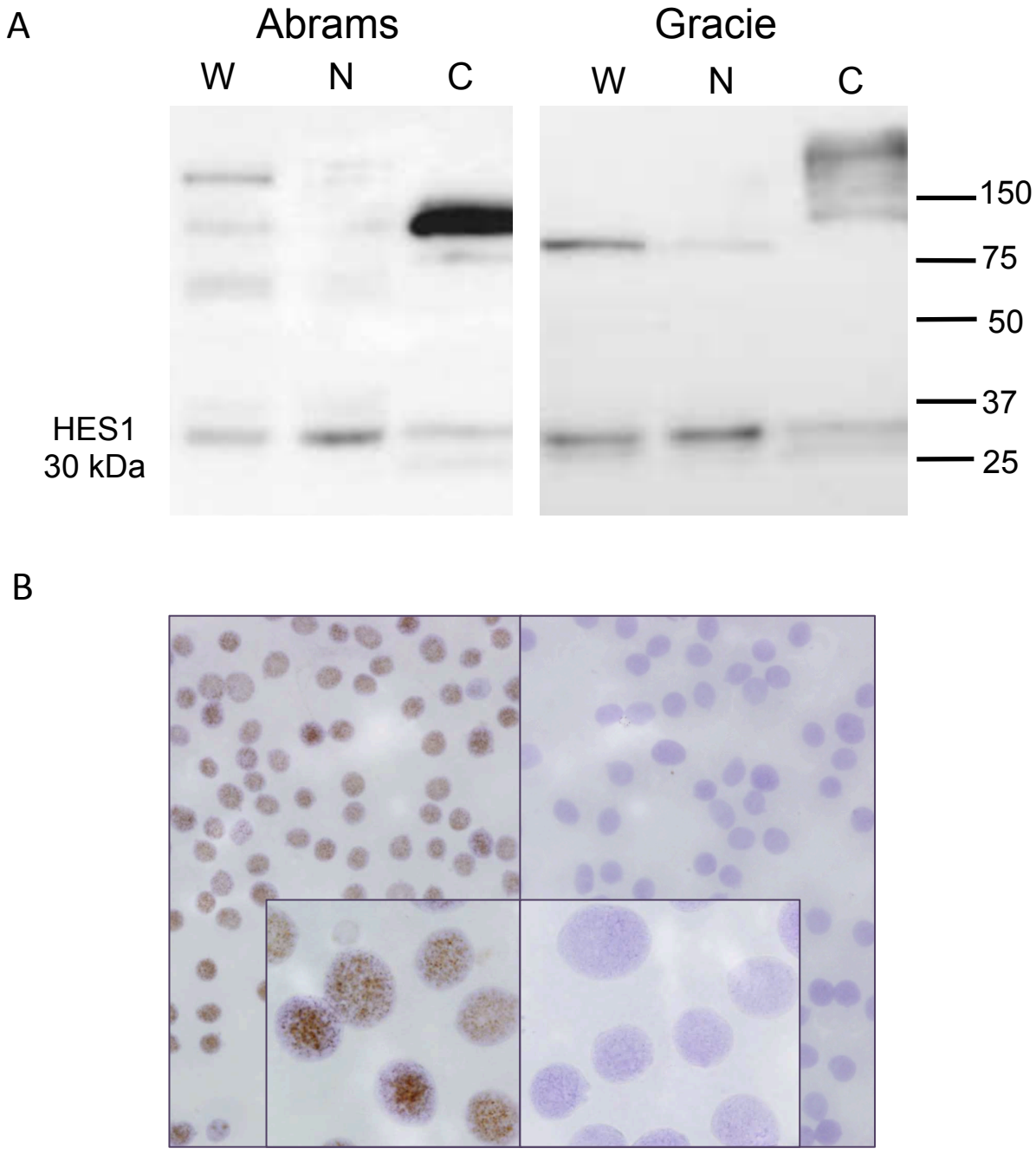


Figure 2.4. Western blot and immunocytochemistry (ICC) results assessing HES1 expression in canine osteosarcoma cells. (A) Western blot analysis of whole cell (W), nuclear (N) and cytoplasmic (C) fractions of canine osteosarcoma Abrams and Gracie cell lines. A 30 kDa band (HES1) is present in whole cell and enriched in extracted nuclear lysates. Larger non-specific bands are enriched in the cytoplasmic fractions. Equal amounts of total protein were loaded in each lane. (B) ICC shows nuclear staining for HES1 in canine OSA cells (Gracie).

Panel on the right is the secondary-only negative control. Photomicrographs were taken at 20x and 100x (oil, inset) magnification; haemotoxylin counterstain.

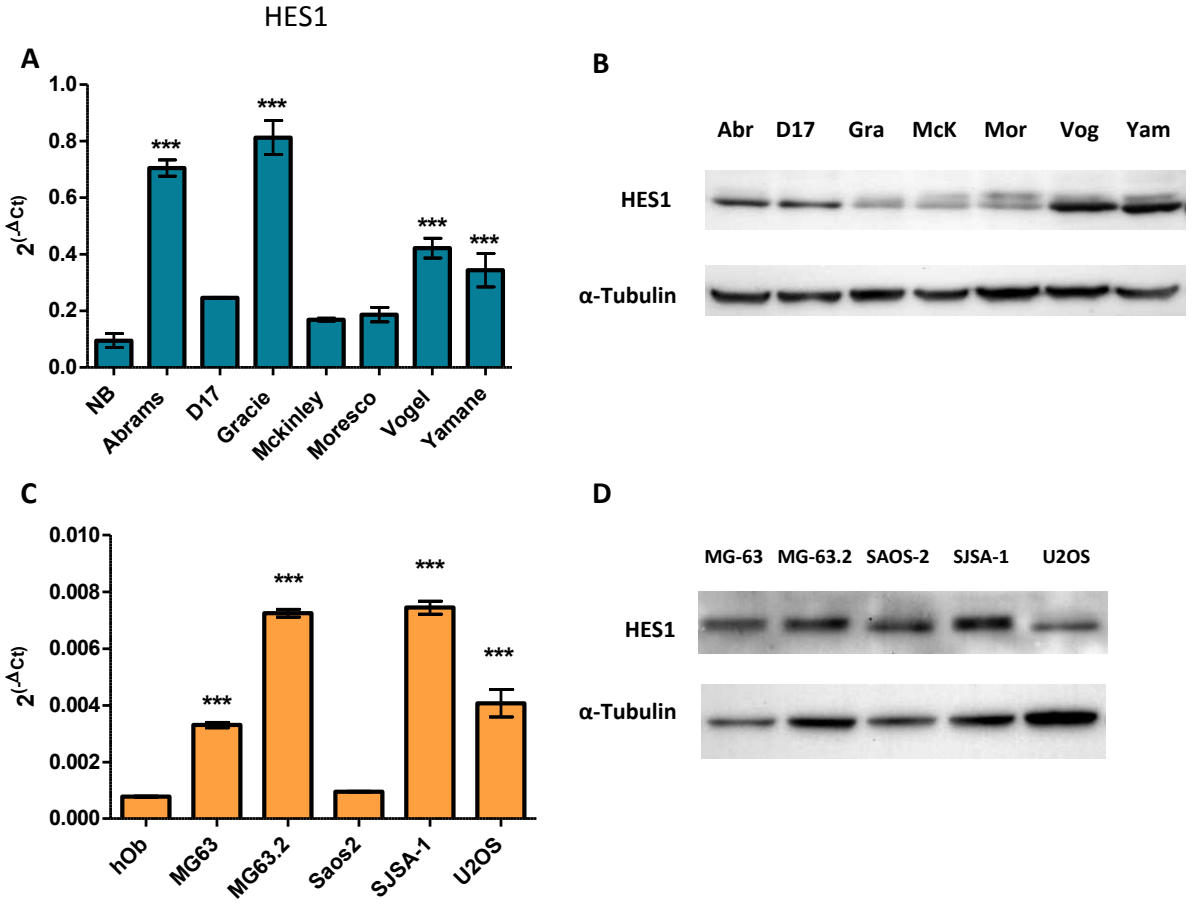


Figure 2.5. **Expression of *HES1* mRNA and protein in human osteosarcoma cell lines.** *HES1* mRNA in canine OSA cell lines and normal canine bone tissue (NB) expressed as $2^{(-\Delta CT)}$ normalized to *HPRT1* (A). *HES1* mRNA in human OSA cell lines and normal human osteoblasts expressed as $2^{(-\Delta CT)}$ normalized to the geometric mean of *RBS15, GAPDH, 18SrRNA,* and *HPRT1* (C). Data are graphed as mean ± SEM, *** P<0.001, ** P<0.01, Two-way ANOVA with Dunnett's Multiple Comparison Test. (B and D) Western blot shows characteristic distinct HES1 band at 30kDa. Blot was stripped and re-probed with an antibody against α-tubulin to serve as a protein loading control.

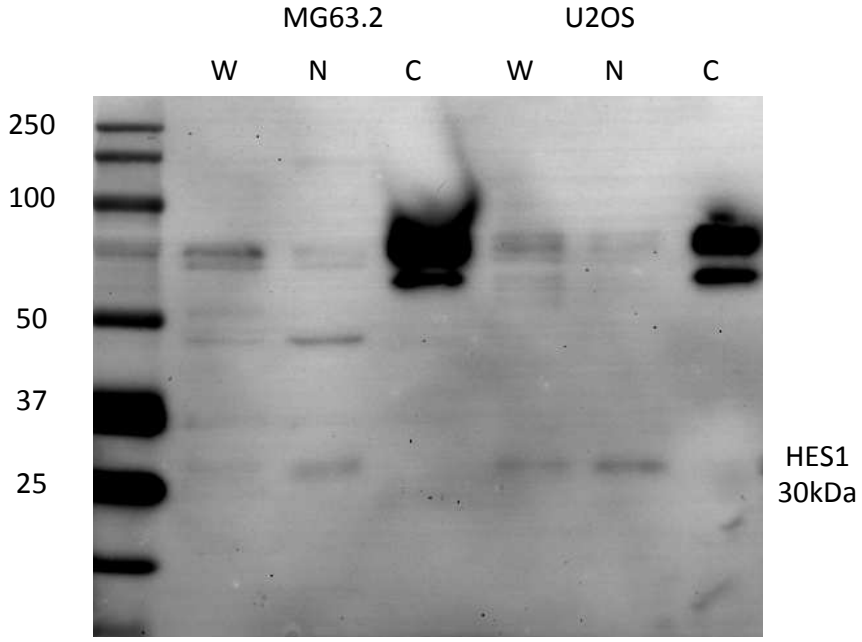
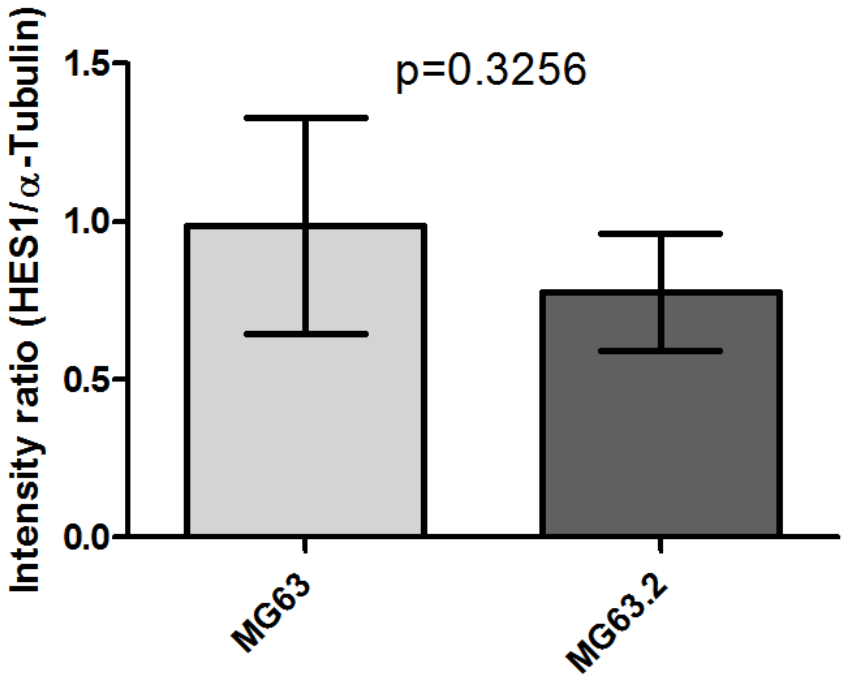


Figure 2.6: Western blot of MG63.2 and U20S whole cell, nuclear and cytosolic fractions for HES1. A distinct band at 30 kDa is present in both MG63.2 and U2OS human OSA whole cell (W) and is enriched in nuclear extract (N) lysates. Larger non-specific bands predominate in the cytoplasmic fraction (C). Equal amounts of total protein were loaded in each lane.

HES1 mRNA and protein expression varied between cell lines in both canine and human OSA cells (Figure 5). For human cell lines mRNA expression was similar to that previously published [24, 25]. In general, *HES1* mRNA expression was increased in canine cell lines relative to normal canine bone tissue (Figure 5A) and in human OSA cell lines relative to human osteoblasts (Figure 5C). Western blot analysis showed a characteristic band at 30 kDa with variable expression between cell lines (Figure 5B and 5D). Interestingly, the metastatic subline of MG63 cells, MG63.2, exhibited elevated levels of mRNA compared to the MG63 line, but protein expression was not significantly different between the two lines (Figure 2.7).



Fgure 2.7: HES1 protein expression is not significantly different between MG63 and MG63.2 cell lines. HES1 band intensity normalized to α-tubulin loading control. Bars represent mean +/- standard deviation from four independent experiments. Standard unpaired 2-tailed t-test was used to compare mean HES1 band intensity ratios for MG63 and MG63.2 Western blot.

We validated immunoreactivity using FFPE human placenta and found positive strong nuclear and cytoplasmic staining of placental macrophages (Hafbauer cells), moderate nuclear +/- cytoplasmic staining of stromal cells and light nuclear staining of endothelial cells consistent with Notch activity in placenta reported by Herr et al. [41]. Staining of additional canine control tissues revealed positive punctate to diffuse intranuclear staining of pancreatic cells, endothelial cells and subsets of pulmonary epithelial cells as described in human literature [42-44] (Figure 2.8). Addition of a blocking peptide specific for the epitope targeted by our antibody eliminated all staining (data not shown). Immunocytochemistry of canine OSA cells (Gracie) showed

diffuse nuclear staining consistent with the specific 30 kDa protein identified in the nuclear lysate by western analysis (Figure 2.9B).

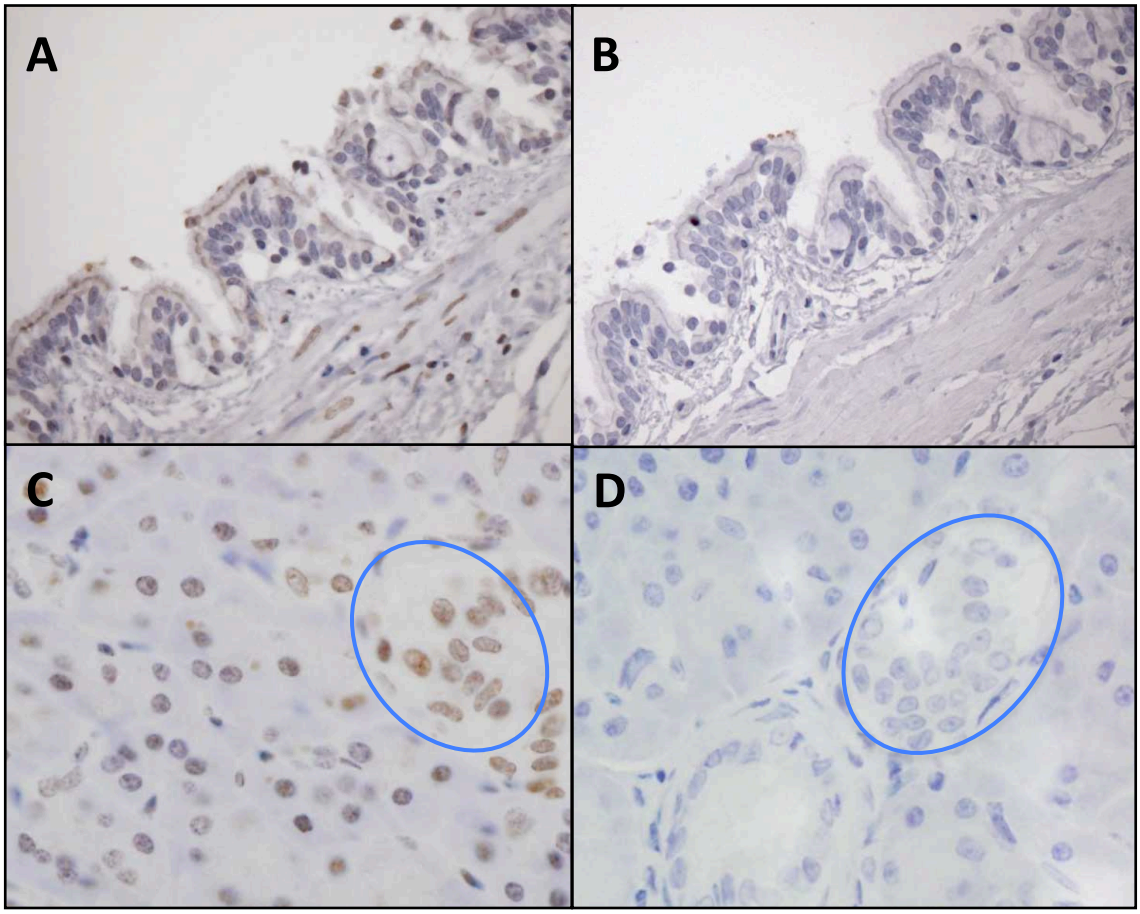


Figure 2.8: HES1 immunohistochemistry of control canine tissues. Variably intense nuclear staining is present in bronchiolar epithelial cells (A) and in both exocrine and endocrine (islets cells, blue circle) pancreatic cells (C). B and D are the negative controls. All photomicrographs were taken at 40x magnification; haematoxylin counterstain.

Increased immunohistochemical HES1 staining is associated with increased disease free interval

Once we established that the RabMAb anti-human HES1 antibody provided specific targeting of HES1 protein in human cultured cells and FFPE tissues with good cross-reactivity in canine samples, we performed immunohistochemistry using canine primary OSA samples. Of

the 20 tumor samples from the canine DFI>300 and DFI<100 tumor groups, 14 were scored as described in the methods (Figure 2.9). For six samples, IHC was not possible due to loss of tissue during processing or poor quality/quantity of staining/tissue present. All OSA samples evaluated with immunohistochemistry had variable positive staining for HES1 both across tumors and within tumors. The staining pattern of tumor cells was predominantly nuclear with diffuse cytoplasmic staining less common. The median HES1 reactivity score was 3 (range, 1 to 9). Of the 6 tumors from dogs with DFI>300 days, 83.3% (n=5) had a score of greater than 3, compared to only 25.0% (n=2) of the 8 tumors from dogs with DFI<100 days (Table 2.2). Consistent with our RT-qPCR results, average HES1 immunohistochemical staining was lower in tumors from dogs with DFI<100 days, but because of low power did not reach statistical significance (Figure 2.10).

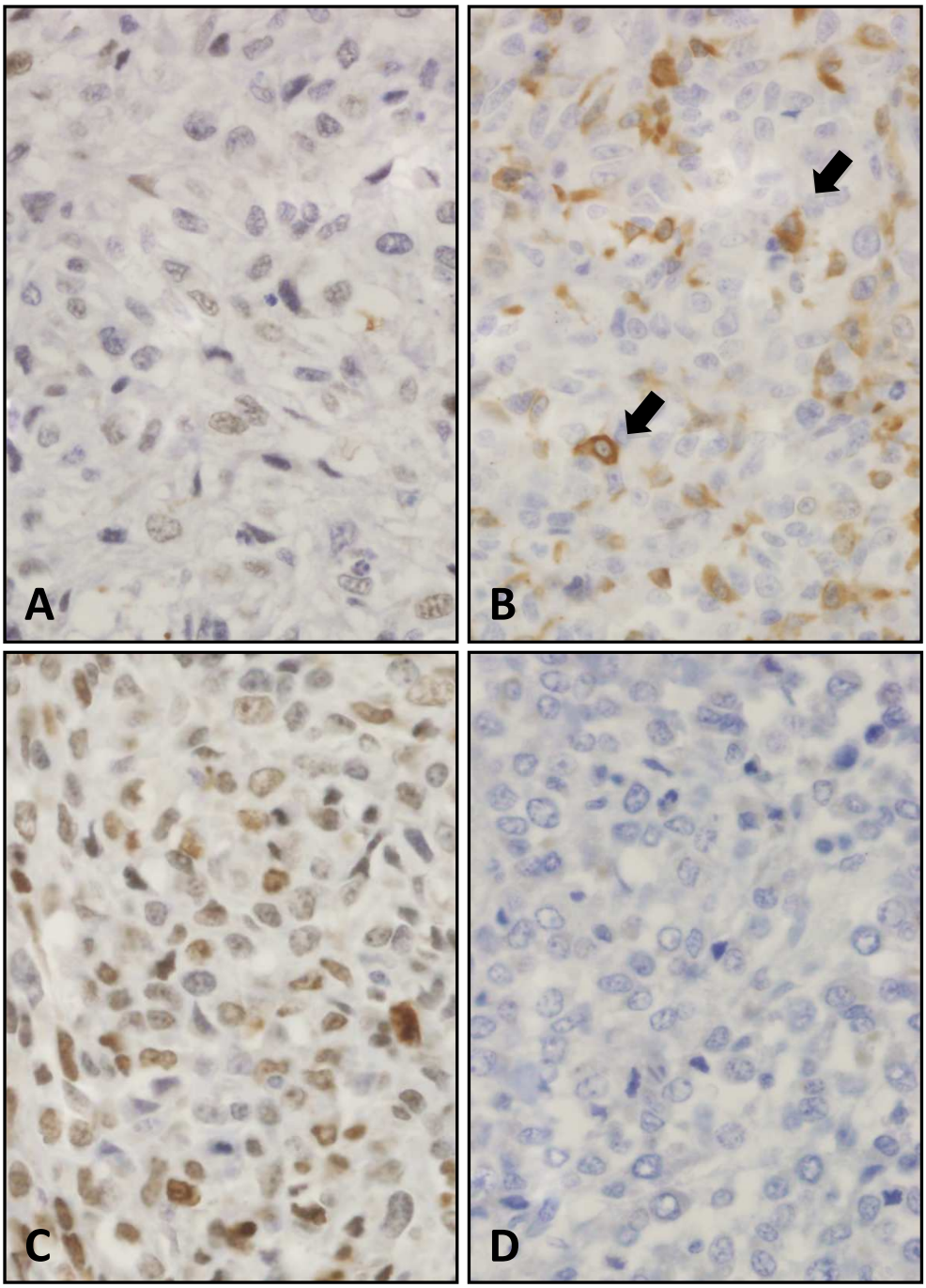


Figure 2.9. Immunohistochemical analysis of nuclear HES1 protein expression in canine osteosarcoma. Examples of low (A and B, score 2) and high (C, score 6) nuclear HES1 expression in canine osteosarcomas (D is a negative control treated only with secondary antibody). Panel B shows example of a field from a low scoring tumor (based on nuclear staining) that includes scattered strong cytoplasmic staining (arrows). All photomicrographs were taken at 40x magnification; haematoxylin counterstain.

Table 2.2. Summary of data for dogs with DFI>300 and DFI<100 days, including HES1 immunohistochemistry score. DFI=disease free interval, Dx=diagnosis, MC=male castrated, FS=female spayed, P=proximal, D=distal, H=humerus, R=radius, T=tibia, Total Score is product of scores for %cells staining and staining intensity.

Breed	Age at Dx (yrs)	Sex	Tumor Loc	DFI (days)	Avg % Stain	Avg Stain Intensity	Total Score
Greyhound	4.4	MC	PH	40	1	1	1
Rottweiler	5	MC	DF	69	3	3	9
Greyhound	7	MC	DF	77	2	1	2
Mix	9	FS	T	90	2	1	2
Greyhound	8	FS	PT	94	1	2	2
Labrador	10.2	FS	DH	95	3	3	9
Mix	8.8	MC	DF	97	2	1	2
Golden	10.8	MC	PH	97	2	1	2
Mix	7.6	FS	DR	307	2	2	4
Greyhound	7.1	MC	PH	467	1	1	1
Mix	12.4	MC	DR	694	3	3	9
Malamute	10.1	FS	DR	734	3	2	6
Labrador	8.7	MC	T	787	3	3	9
Golden	8	FS	DR	885	3	2	6

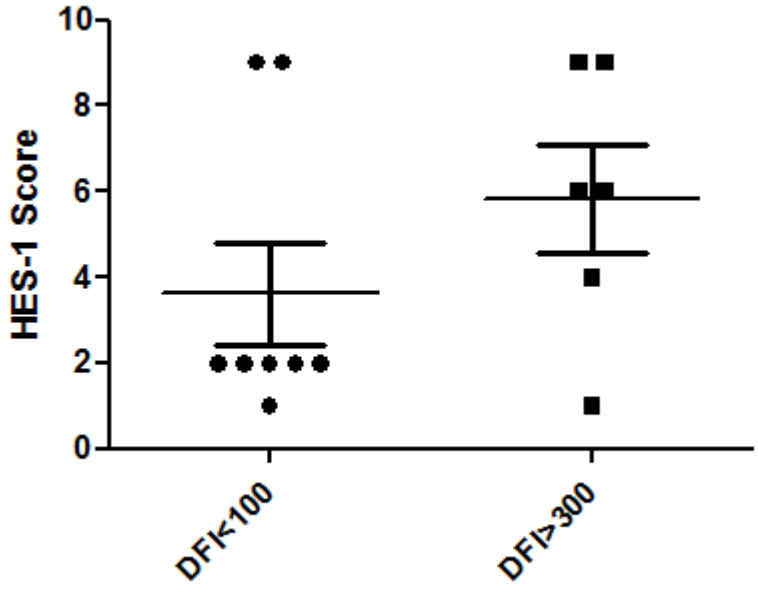


Figure 2.10 HES1 immunoreactivity in canine osteosarcomas from DFI<100 and >300 groups. Immunoreactivity scores of nuclear HES1 protein expression in tumor sections from DFI<100 day (filled circles, n=8) and DFI>300 day (filled squares, n=6) groups. Horizontal line and error bars are mean ± SEM (p=0.1026).

To further assess the utility of HES1 protein expression as a prognostic biomarker, we performed IHC on 61 primary canine OSA tissues from a subset of dogs in a previously reported prospective clinical trial [33]. Demographic information for this patient population is supplied in Additional file 7. IHC scores were assigned as described in materials and methods. HES1 was expressed in all tumors with a median HES1 immunoreactivity score of 4 in this population (range, 1 to 9). The overall median DFI was 168 (range 43 to 1,393+ days). The median DFI in dogs with a high HES1 immunoreactivity score (≥4) was 258 days compared to 155 days in dogs with a low HES1 immunoreactivity score (< 4) (p=0.0023; Figure 2.10). Univariate analysis identified HES1, bone-specific alkaline phosphatase (BALP) activity, histologic grade, percent

necrosis and mitotic index as potential predictors of DFI (Table 2.3, $p<0.1$). Upon multivariate analysis, HES1, percent necrosis and mitotic index retained statistical significance ($p = 0.029$, 0.002 and 0.005 respectively; Table 2) as independent predictors of DFI. In summary, consistent with our prior RT-qPCR analysis, increased HES1 expression was identified as an independent prognostic biomarker for increased disease free survival in 61 canine OSAs treated by amputation and chemotherapy.

Table 2.10: Summary demographic data for 61 canine patients from a previously reported clinical trial [33].

Demographic Summary			
Median Age yrs (range)		8	(2-13)
Median Wt kg (range)		34.09	(16-64)
		n	%
sex	%NM	24	39.34
	%MI	3	4.92
	%SF	33	54.10
	%FI	1	1.64
breed	Mixed	16	26.23
	GR	11	18.03
	Rott	8	13.11
	GH	6	9.84
	Lab	5	8.20
	GSD	3	4.92
	Other	12	19.67
Tumor loc	humerus	22	36.07
	tibia	16	26.23
	radius	13	21.31
	femur	10	16.39
tumor grade	I	15	24.59
	II	24	39.34
	III	11	18.03
	no grade given	11	18.03

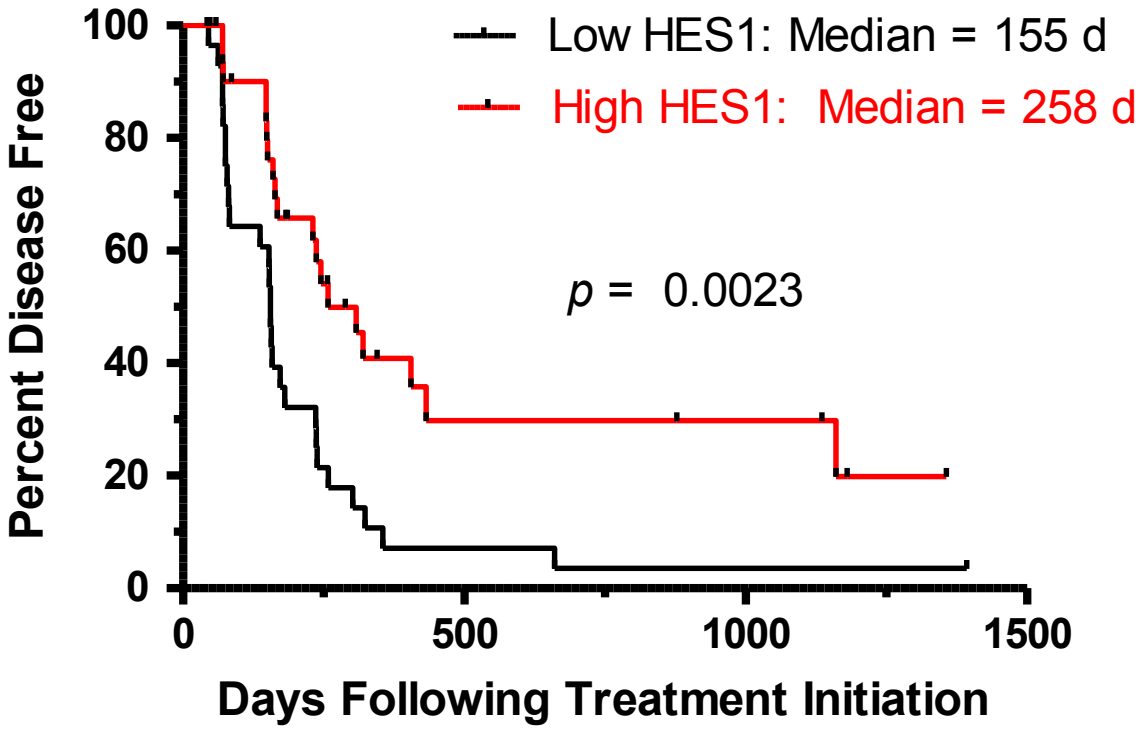


Figure 2.11. High HES1 immunoreactivity score correlates with lower histologic grade and improved outcome in canine osteosarcoma. Kaplan-Meier plot of disease free interval based on HES1 immunoreactivity score. Dogs with high HES1 scoring tumors (score ≥ 4, n=28) had a statistically significantly longer disease free interval than dogs with low HES1 scoring tumors (score < 4, n=33) (p=0.0023, Log Rank test).

Table 2.11. Results of univariate/multivariate analysis of factors associated with clinical outcome. DFI=disease free interval, BALP=bone-specific alkaline phosphatase

Univariate Analysis

		Median DFI (d)	HR	P	95% CI
HES1 Score	<4	155	0.388	0.0023	0.211-0.712
	≥4	258			
BALP	<36	273.5	1.871	0.0377	1.036-3.378
	≥36	157			
Necrosis %	<20%	239	1.799	0.098	0.897-3.609
	≥20%	168			
Mitotic Index	<54	258	3.234	0.0163	1.241-8.428
	≥54	153			
Grade	1 or 2	308	15.43	<0.0001	4.243-56.07
	3	75			

Multivariate Analysis

	HR	P	95% CI
HES1 Score	0.775	0.029	0.616-0.975
Necrosis %	1.032	0.002	1.012-1.053
Mitotic Index	1.033	0.005	1.01-1.057

DISCUSSION

Expression of *HES1* mRNA is frequently utilized as an indicator of Notch activity and Notch/HES1 activation has been implicated in a variety of human cancers with oncogenic activity in some tumor types and tumor suppressor activity in others [17-20, 24-27]. The goals of this study were to evaluate expression of Notch receptors and signaling mediators, HES1 and HEY1, in canine OSA samples from dogs with DFI>300 days and DFI<100 days as well as samples of matched OSA and normal bone to explore associations with OSA progression and patient outcome. Gene array analysis focusing on 51 Notch/HES1 associated genes identified elevated expression of Notch signaling mediators in tumors relative to normal bone. We

confirmed a statistically significant elevation of *NOTCH2*, *HEY1*, and *HES1* mRNA expression in OSA when compared with normal bone. Interestingly, we did not find elevated *HES1* expression in the most aggressive OSA when comparing good and poor responders, but instead identified a statistically significant association between high *HES1* mRNA and protein expression and longer DFI following standard treatment. Further, the gene array analysis of Notch/HES1 associated genes and RT-qPCR analysis of *NOTCH1, NOTCH2* and *HEY1* showed no significant differences in expression between the DFI groups. Overall, our findings indicate that alterations in Notch signaling occur during the development of canine OSA, but mechanisms that do not alter HES1 expression may drive the most aggressive tumors.

The oncogenic role of Notch signaling in OSA in humans is supported by previous studies [24-26]; however, the specific role of HES1 is less clear. A common finding regarding HES1 expression between these previous studies and ours is the variability of expression within human and canine OSA cells and tumors (please note for references 24 and 28, that data from experiments done using the OS187 or COL cell lines should be viewed with caution due to a recent disclosure that these cells are not OSA cells) [24-26, 28]. For example, *HES1* mRNA expression in tumors relative to normal bone was elevated in 5 of 9 canine tumors relative to matched normal bone samples in our study (Figure 3B) and 6 of 10 human tumors in the Tanaka study [25]. There is also disagreement among studies as to which Notch receptors and target genes are functionally significant in OSA. Zhang et al. provided evidence that increased Notch1 activity and Notch1-induced expression of HES1 specifically are associated with invasion and metastasis in two OSA cell lines, the low HES1 expressing SAOS2 parental line and the metastatic, high HES1 expressing LM7 sub-line [24]. Inhibition of Notch signaling by a gamma-secretase inhibitor suppressed LM7 OSA cell invasion, but had no effect on proliferation or

tumorigenesis; whereas induced expression of intracellular cleaved Notch1 (ICN1) or HES1 in the SAOS2 line increased invasiveness. Tanaka et al. identified elevations of *NOTCH2* and *HEY1* mRNA in human OSA biopsy specimens relative to normal bone, but *NOTCH1* and *HES1* mRNA expression was not consistently elevated. In the same study, treatment of OSA cells and tumors grown in nude mice with a gamma-secretase inhibitor reduced proliferation through a G1 block [25]. Differing results in these two studies may be due to different samples studied (tumor vs. cells) and/or the use of different gamma-secretase inhibitors. Our RT-qPCR data suggests that *NOTCH2* and *HEY1* may be primary mediators of Notch signaling in canine OSA as well. Interestingly, Zhang et al observed both elevated *HES1* mRNA expression [24] and elevated HES1 protein expression [28] in the LM7 metastatic sub-line relative to the SAOS2 parent line. We also observed an increase in *HES1* mRNA expression in the MG63.2 metastatic sub-line relative to the MG63 parent line. However, western blot analysis identified similar levels of HES1 protein in the MG63 and MG63.2 lines suggesting that post-transcriptional regulation may be important.

Studies exploring the relationship between HES1 expression and patient outcome in OSA are limited. Our RT-qPCR results (n=20) revealed significantly increased *HES1* mRNA expression in canine OSA from dogs with a longer DFI compared to those with a short DFI. This relationship was confirmed by immunohistochemical examination of HES1 protein in a larger dataset (n=61). These results conflict with those of Hughes who conducted a RT-qPCR study using tissue from 16 primary OSAs that suggested lower *HES1* mRNA expression may be associated with a better prognosis [27]. Discrepancy from our results may be due to differing sample sizes, different measurements of outcome and different outcome groupings. Despite evidence of strong molecular similarities of canine and human OSA and high conservation of

Notch/HES1 between species, there is also the possibility that canine tumors may exhibit different characteristics than their human counterparts. Until similar studies to evaluate nuclear immunoreactivity as a measure of protein expression are carried out in human tumors, no firm conclusions regarding possible differences in canine and human OSA with respect to HES1 expression can be made.

Previous studies examining HES1 expression in other cancers or during development provide candidate mechanisms for reduced HES1 expression in the presence of elevated Notch signaling: uncoupling of HES1 from Notch signaling, cell cycle regulation of HES1 expression, and post-transcriptional regulation. HES1 expression has been reported to be uncoupled from Notch signaling in Ewing's sarcoma [15] and stimulation of HES1 transcription by sonic hedgehog (Shh) pathway occurs in mesodermal and neural stem cells [6 – 8]. Using RT-qPCR analysis, we identified significantly decreased SMO mRNA expression ($p<0.05$) in the DFI<100 tumors compared to the DFI>300 tumors [32] suggesting that reduced HES1 expression in aggressive canine OSA might reflect a loss of Shh signaling. HES1 expression oscillations are both observed and necessary for cell cycle progression during neuronal development [45]; aggressive OSA tumor cells may utilize HES1 oscillatory patterns to manipulate the cell cycle and optimize their ability to metastasize and/or resist chemotherapy. Finally, several miRNAs have been shown to regulate HES1 (miR-124 and miR-23b) [46, 47] and may contribute to altered HES1 expression in OSA cells and tumors.

In addition, HES1 protein may exhibit specific functions depending on its phosphorylation status and binding partners. Kannan et. al. found that interactions with HES1 stimulates PARP1 activation and cleavage, ultimately resulting in apoptosis in B-ALL (overall a tumor suppressor role for HES1) [20]. Further, in neuronal development, Ju et al. showed that

HES1 interactions with phosphorylated PARP1 released HES1 from the HES1/groucho/TLE repressor complex and, upon HES1 phosphorylation, led to association with a co-activator complex, changing the role of HES1 from a transcriptional repressor to a transcriptional activator [48]. In bone development, via inhibition of RUNX2, Notch activity maintains a population of committed osteoblast precursors [49, 50]. Interestingly, several studies also show that HES1 binding stabilizes and activates RUNX2 protein; thus, HES1 has been shown to both inhibit and enhance the activity of RUNX2 [49, 51]. Additional studies exploring the phosphorylation status and binding partners of HES1 may provide a better understanding of these interactions in OSA.

Conclusions

The results of the current study support the association of Notch pathway activation with the proliferative response of OSA. However, reduced HES1 expression in the most aggressive tumors despite the elevated expression of other Notch signaling effectors and targets indicates that HES1 is not an ideal sole surrogate marker of Notch signaling. Further, these findings suggest that additional mechanisms beyond Notch signaling may contribute to the aggressive phenotype of these tumors. Studies to define the role of Notch signaling in OSAs is warranted as inhibitors for this and other developmental pathways that impinge on HES1 are currently in clinical trials for the treatment of a variety of human cancers (summarized in Sang et al.) [52]. Research in this area may reveal important regulatory mechanisms contributing to metastasis and therapeutic resistance in both canine and human OSA. While we found that HES1 expression was not consistently linked to Notch signaling in canine OSA, our study has determined that reduced HES1 expression serves as an independent prognostic biomarker.

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Chapter 3

Tumor and serum-derived miRNA expression changes associated with poor outcome in canine osteosarcoma

SUMMARY

MicroRNAs (miRNA) are small non-coding RNA molecules involved in post-transcriptional gene regulation. Deregulation of miRNA expression occurs in cancer and miRNA expression profiles have been associated with diagnosis and prognosis in many human cancers. Osteosarcoma (OS), an aggressive primary tumor of bone affects approximately 400 children or adolescents and 10,000 dogs each year. Though survival has improved with addition of chemotherapy, approximately 1/3 of human patients and up to 80% of canine patients will succumb to metastatic disease. Reliable prognostic markers are lacking for this disease.

MicroRNAs are attractive targets of biomarker discovery efforts due to their increased stability in easily obtained body fluids as well as within fixed tissue. These features make this class of biomarker ideal for the veterinary care setting where the stringent sample handling requirements necessary for mRNA-based diagnostics is not always practical. Previous studies in our laboratory demonstrated dysregulation of genes in aggressive canine OS tumors that participate in miRNA regulatory networks reportedly disrupted in OS or other cancers. We utilized RT-qPCR in a 384-well plate system to measure relative expression of 190 miRNAs in 14 canine tumors from two cohorts of dogs with good or poor outcome (disease free interval >

300 days or < 100 days respectively). Differential expression analysis in this subset guided selection of candidate miRNAs in a tumors and serum samples from larger groups of dogs.

We ultimately identified a tumor-based 3-miR Cox proportional hazards regression model and a serum-based 2-miR model each able to distinguished patients with good and poor prognosis via Kaplan-Meier analysis with the log rank test. Additionally, we integrated miRNA and gene expression data to identify potentially important miRNA-mRNA interactions disrupted in canine OS. Applying this methodology with miRNAs differentially expressed between OS tumors and normal bone, we confirm miRNA disruption likely contributes to the dysregulation of Notch-related genes identified in Chapter 1. Interestingly, integrated analyses of miRNAs in the 3-miR predictive model and disrupted genes from previous expression studies suggest contribution of the primary tumor microenvironment to the metastatic phenotype of aggressive tumors.

INTRODUCTION

Despite increased survival in OS patients resulting from the addition of chemotherapy to standard treatment protocols, only about 25% of canine OS patients will survive longer than a year.[1] New treatment strategies are needed to manage this disease and will likely include integration of targeted therapies with standard chemotherapeutics in an individualized medicine setting. To facilitate this effort, biomarkers of disease progression and response to treatment are needed to 1) optimize the stratification of patients into groups most likely to benefit from various treatments and 2) identify targets for development of novel therapeutics.

In the previous chapter, gene expression studies identified activation of the Notch signaling pathway in OS, but suggested Notch-independent changes resulted in low HES1 expression in the most aggressive tumors. Other studies in our laboratory identified upregulation of IGF2BP1, a known target of the let-7 tumor suppressor family of miRNAs that also contains alternate polyadenylation sites within an approximately 6 kb 3' untranslated region (UTR).[2-4] We hypothesized that the disconnect between HES1 and Notch pathway activation, as well as the escape of IGF2BP1 from inhibitory mechanisms present in normal adult cells, likely involved disruption of post-transcriptional regulation by miRNAs.

MicroRNAs are small non-coding RNAs that fine-tune the regulation of gene expression in multiple cellular processes involved in development and maintenance of homeostasis. In general, miRNAs suppress expression of their target genes, and it is estimated that half of mammalian genes are subject to miRNA regulation via 3' UTR binding sites.[5, 6] Since a 2002 report from the Croce laboratory, the involvement of miRNA dysregulation in cancer has been well established.[7] Molecular genomic techniques such as cDNA microarrays and next generation sequencing have been adapted to facilitate miRNA expression biomarker and novel target discovery efforts.[8, 9]

MicroRNAs are attractive molecules for biomarker discovery efforts due primarily to increased stability in biologic fluids and in formalin fixed tissues compared to other RNA molecules.[10-12] These features exemplify the clinical utility of miRNA, particularly in the veterinary setting where stringent sample collection and storage requirements necessary for analysis of mRNA are not always practical or affordable. A handful of studies in the human literature have identified associations between miRNA expression and outcome in OS, including studies utilizing paraffin-embedded formalin-fixed tissues and blood fluids.[13-15] Given the

molecular similarities between canine and human OS, we undertook studies to explore the hypothesis that cancer-associated miRNAs would be measurable in tumor and serum and associated with outcome.

The first aim of the studies conducted in Chapter 3 was to identify candidate biomarker miRNAs differentially expressed in tumors from different outcome groups and in all tumors relative to normal bone. Candidate miRNAs were measured in a larger group of tumors and a similarly sized set of serum samples to determine associations between miRNA expression changes and patient outcome. Finally, pathway and miRNA target prediction analyses were used to integrate miRNA and gene expression data to identify potential miRNA-gene regulatory networks important for OS progression.

METHODS

Selection of tumors and candidate miRNAs

Tumors in disease free interval (DFI) cohorts from dogs with DFI > 300 days or DFI <100 days were largely the same as those used for studies described in Notch expression studies (Chapter 2). Three new tumors were added to replace samples that had been used up in previous studies, one from a patient with DFI<100 and two from patients with DFI < 300. Criteria for selection were as previously described. Table 3.1 shows updated patient data for this group of tumors. Similarly, the seven normal bone samples included three of the same normal bone tumors used previously and four new samples.

MC=male castrated, FS=female spayed, MI=male intact

Table 3.1. **Patient information for 14 tumors (7 from dogs with DFI <100 days and 7 from dogs with DFI > 300 days).** MC=male castrated, FS=female spayed, MI=male intact

Unique ID	DFI (days)	Age at Dx (yrs)	Sex	Breed	Tumor Loc	Chemotherapy
T1	97	8.8	MC	Mix	Femur	Doxorubicin
T2	80	9.4	MI	Rhodesian	Humerus	Carboplatin
T3	885	8	FS	Golden	Radius	Carboplatin
T4	356	10.4	MC	Mix	Radius	Doxorubicin
T5	40	4.4	MC	Greyhound	Humerus	Doxorubicin
T6	384	11.5	FS	Mix	Femur	Cisplatin
T7	90	9	FS	Mix	Tibia	Cisplatin
T8	467	7.1	MC	Greyhound	Humerus	Doxorubicin
T9	95	10.2	FS	Labrador	Humerus	Cisplatin
T10	605	7.1	FS	Greyhound	Femur	Doxorubicin
T11	77	7	MC	Greyhound	Femur	Carboplatin
T12	466	6.9	MC	Rottweiler	Radius	Doxo & Carbo
T13	94	8	FS	Greyhound	Tibia	Doxo & Carbo
T14	307	7.6	FS	Mix	Radius	Cisplatin

Sixty new tumors were selected from the Colorado State University (CSU) Flint Animal Cancer Center's (FACC) tissue archive as described in Chapter One with the additional criteria that cases had to be at least a year old; in other words, the original diagnosis had to have been made at least one year prior. Additionally, tumors needed to have matched serum or plasma samples available in the archive. Of these sixty cases, 33 were selected for miRNA expression analysis after extraction of RNA and quality checks (Table 3.2). Three tumors overlapped with tumors in the DFI cohort, though an independent tumor sample from the same patient was processed in each group. This data set is subsequently referred to as the COS33 data set.

Table 3.2. Patient data for 33 canine OS patients. MC=male castrated, FS=female spayed, MI=male intact

Unique ID	DFI (days)	Age at Dx (yrs)	Sex	Breed	Tumor Location	Chemotherapy
6B	97	8.8	MC	Mix	Femur	Doxorubicin
11B	252	7.5	FS	Mastif	Radius	Doxorubicin
25B	466	6.9	MC	Rottweiler	Radius	Doxo & Carbo
60B	427	6.5	MI	Old Eng Sheep	Tibia	Doxo & Carbo
41B	80	9.4	MI	Rhodesian	Humerus	Carboplatin
5B	120	6.6	MC	Mix	Radius	Doxo & Carbo
4B	406	9.4	MC	Mix	Radius	Doxorubicin
7B	150	10.8	FS	Golden Ret	Humerus	Doxorubicin
9B	232	6.6	FS	Dane	Humerus	Doxo & Carbo
51B	34	6.1	F	Dane	Tibia	Carboplatin
17B	475	9.5	MC	Husky	Femur	Doxo & Carbo
52B	95	11.8	FS	Mix	Humerus	Doxorubicin
18B	151	6.9	MC	Mix	Femur	Doxo & Carbo
20B	937	10.4	MC	Australian Shep	Tibia	Doxo & Carbo
22B	605	7.1	FS	Greyhound	Femur	Doxorubicin
23B	20	9.8	MC	Germ Short Point	Radius	Carboplatin
24B	218	5.1	MC	Pyrenees	Radius	Doxo & Carbo
54B	127	4.8	MC	Pyrenees	Radius	Doxo, Carbo, & Cisp
55B	91	6.1	FS	Greyhound	Humerus	Doxo & Carbo
30B	296	11.4	MC	Labrador Ret	Scapula	Carboplatin
32B	299	8.0	FS	Dane	Radius	Carboplatin
33B	246	7.9	MC	Rottweiler	Tibia	Doxorubicin
34B	64	7.5	FS	Rottweiler	Humerus	Doxo & Carbo
35B	190	8.5	MC	Labrador Ret	Radius	Doxo & Carbo
36B	75	5.3	MI	Leonburger	Tibia, tallus	Doxo & Carbo
59B	392	11.1	FS	Mix	Tibia	Carboplatin
37B	132	4.2	FS	Saint Bernard	Radius	Carboplatin
38B	256	10.8	MC	Great Pyrenees	Radius	Doxo & Carbo
40B	216	8.5	MC	Rottweiler	Femur	Carboplatin
61B	97	8.7	FS	Flat-Coated Ret	Radius	Doxo & Carbo
42B	77	7.2	MC	Labrador Ret	Radius	Doxo & Carbo
43B	756	8.9	FS	Coonhound	Radius	Carboplatin
44B	376	7.1	MC	Labrador Ret	Humerus	Carboplatin

Thirty-four of the seventy-one eligible canine patients in both DFI cohort and COS33 tumor sample groups had archived serum samples. Serum samples were chosen as there were more of those and it was decided not to use a mixture of different sample types (i.e. serum and plasma) in hopes of eliminating sample type as one source of variability. There was overlap of 24 patients for which both serum and tumor samples were ultimately used for miRNA expression analysis experiments.

Total RNA Isolation, Quantification, and Quality Assessment (Tissues)

RNA was extracted from frozen samples using a freeze fracture device, followed by homogenization and separation of RNA from DNA and protein fractions using TRIzol® Reagent (Life Technologies, Grand Island, NY). The freeze fracture device and the samples were placed in liquid nitrogen to chill for 15-20 minutes. Approximately 1cm^3 of tumor tissue (depending on amount of available tissue present) and up to 4cm^3 of normal metaphyseal bone were used for RNA extraction. Tissue was pulverized in the freeze fracture device and, using a chilled spatula, transferred into TRIzol (2mL/cubic cm of tissue) in 15ml conical tubes. For tissue samples less than 1 cm^3, 1 ml of TRIzol reagent was placed in the 1 cm diameter opening of the freeze fracture device before loading the tissue sample and crushing the tissue sample. The crushed pellet was then transferred to a 15 ml conical tube with the 1 ml of TRIzol reagent. The tissue/TRIzol mixture was homogenized at medium to high speed for one minute. Tubes were capped and transferred to ice. The homogenizer was washed in a series of five washes between each sample: deionized water, RNase Away (Sigma-Aldrich, St. Louis, MO), deionized water, 100% ethanol, deionized water. Homogenized samples were gently shaken, centrifuged for one minute at 2000 RPM, and incubated for 5 minutes at room temperature. The supernatant was

collected into two 1.5 ml tubes and carried forward using the TRIzol reagent manufacturer's protocol for RNA extraction.

After resuspension of the extracted RNA pellet in nuclease free water, the mirVana™ miRNA extraction kit (Life Technologies, Grand Island, NY) was used for additional RNA purification. RNA was eluted in 50 µl nuclease free water and treated in 20 µl batches with DNAse (2 µl 10x DNAse buffer and 2 µl DNAse-I (DNA-free™ kit, Life Technologies)) to eliminate genomic DNA contamination. RNA concentration and purity were determined using the NanoDrop 1000 spectrophotometer (NanoDrop Products (Thermo Scientific), Wilmington, DE). The quality of isolated total RNA was determined by RNA integrity number using a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA) with a RNA 6000 Nano chip. Only samples with RNA integrity number >6 were used. All samples were stored at -80^{o}C.

Total RNA Isolation (Serum)

Archived serum samples stored at -80^{o}C were thawed at room temperature, transferred to RNAse/DNAse Free 2ml microcentrofuge tubes and centrifuged for 5 minutes at 4^{o}C and 16,000 x g. Exactly 200 µl of the supernatant was moved to a fresh 2ml tube for extraction of RNA using the miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA) following the manufacturer's directions. Preset volumes of reagents used and sample containing RNA extracted are shown in Table 3.3 and 1.6x10^{8} copies of synthetic ce-miR-39 mimic was added to each sample prior to addition of chloroform. The minimum recommended volume of RNase-free water (10 µl) was used to elute RNA to maximize RNA concentration.

Table 3.3: **Volumes for reagents and sample used in various steps of RNA isolation using the Qiagen miRNeasy Serum/Plasma kit**.

Reagent/Sample	Volume
Starting sample	200 µl
QIAzol Lysis reagent	1 ml
Chloroform	200 µl
Aqueous phase	600 µl
100% ethanol	900 µl
Rnase-free water	10 µl

Real-Time Reverse Transcriptase Quantitative PCR

cDNA synthesis of small non-coding RNAs was performed using the miScript Reverse Transcription kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly, reverse transcription (RT) was performed in 20 µl reactions containing 1ug total RNA in nuclease free water, 5x miScript RT Buffer (Mg, dNTPs, and oligo-dTprimers), and 1 µl miScript Reverse Transcriptase Mix (poly(A)polymerase and reverse transcriptase). Polyadenylation of miRNA and RT were carried out in parallel by incubating samples at 37^{o}C for 60 minutes, followed by 95^{o}C for 5 minutes (RT inactivation). Generated cDNAs were stored at -20^{o}C until analysis. Quantitative PCR measurements were performed in 384-well PCR plates in a 6 µl reaction containing 2x Quantitect SYBR Green master mix (Qiagen, Valencia, CA), 10 µM miRNA specific forward primer, 10x Universal Reverse Primer (Qiagen, Valencia, CA), 2ng equivalent cDNA, and nuclease free water. Samples were run in duplicate. Non-template controls were included on each plate. Controls using cDNA without reverse transcriptase (NoRT) were run on a separate plate. Primers used are listed in Appendix I. Two miRNAs were eliminated from further analysis, miR-138 and miR-200c due to amplification in NoRT controls.

Modifications to this protocol for measurement of serum miRNA were as follows. cDNA synthesis was carried out in 10 µl reactions containing 2 µl 5x HiSpec Buffer, 1 µl Nucleics mix,

1 µl nuclease free water, and 5 µl total serum RNA. The serum cDNA was diluted 1:10 in nuclease free water and a consistent volume (0.15 µl), rather than a consistent concentration, was included in each 6 µl RT-qPCR reaction. For each tumor or serum RNA sample for which cDNA synthesis was performed a no RT sample was prepared in parallel substituting nuclease free water for RT.

Data Analysis

For analysis of RT-qPCR data from tumor samples, ten miRNAs were selected for potential use as reference genes for normalization based on low variability across samples and between DFI groups. Three commonly used human small RNAs failed to meet this criteria. Both GeNorm[16] and NormFinder[17] were used to identify top candidates and data was normalized to the geometric mean of three high-scoring miRNAs, miR-30a, miR-27b and miR-185. The $2^{-\Delta\Delta Ct}$ method was used for differential expression analysis in 14 tumors. Statistical analysis of survival data was performed using normalized and transformed expression data from 19 miRNAs in 33 tumors and 13 miRNAs in 31 serum samples using a combination of Prism and the coxph and survfit functions from the survival package in R. Associations between miRNA expression levels and DFI were evaluated using Cox proportional hazards linear regression. Multivariable Cox regression was then performed, utilizing both forward and backward stepwise models based on the Akaike information criterion (AIC). Variables with a univariate p-value of <0.25 were included in the multivariate analyses. A risk score was calculated for each sample based on the best multivariate model and the Kaplan-Meier method was used to determine median DFI for low and high risk groups based on the median risk score. Comparison between groups was made with the log rank analysis and a p-value of <0.05 was considered significant.

This analysis pipeline was modified slightly for serum samples. Thirteen candidate miRNA biomarkers selected based on results from the tumor expression analysis as well as candidate serum biomarkers identified in the OS literature. Five miRNAs were selected as potential reference miRs based on typical high expression in human serum (Qiagen whitepaper). Raw Ct values were first adjusted based on expression of the synthetic cel-miR-39.[10, 18] Then two miRs, miR-16 and miR-21 were selected as reference miRs and normalized using a variation of mean-centering termed concordance correlation restricted (CCR) described in Wylie et al.[19] This method was found to be well suited for biofluid samples. The remainder of the analysis was conducted as described for tumor miRNAs.

RESULTS

Differentially expressed miRNAs in tumors from dogs with poor response compared to those with good response are associated with patient outcome.

Expression of 188 miRNAs was measured in 14 tumors, 7 tumors from dogs with disease free interval (DFI) greater than 300 days (good responders) and 7 tumors from dogs with DFI less than 100 days (poor responders) using RT-qPCR. Four miRNAs were differentially expressed in tumors from poor responders relative to those from good responders using a cut-off for significance of $p<0.05$ (Table 3.4). Nineteen miRNAs were selected based on $p<0.1$, fold change > 2.0, as well as biological interest based on human OS studies for additional expression analyses in a larger set of tumors (Table 3.5).

Table 3.4: **Differentially expressed miRNAs in tumors from dogs with poor response (DFI<100 days) compared to tumors from dogs with good response (DFI>300 days).** (P < 0.2, Student's Ttest and ddCt method, shaded rows p < 0.05, bold miRNAs are those selected for additional analyses).

miRName	Ttest p-val	Fold Change	Direction of Exp Δ
miR-26a-5p	**0.0203**	**-2.00**	**down**
miR-142-3p	**0.0281**	**2.77**	**up**
miR-135a-3p	**0.0324**	**-9.76**	**down**
miR-451a	**0.0492**	**4.45**	**up**
miR-30c-5p	**0.0564**	**-1.61**	**down**
let-7c-5p	0.0593	-1.76	down
let-7b-5p	**0.0604**	**-2.07**	**down**
miR-181b-5p	**0.0670**	**-2.20**	**down**
miR-128-3p	**0.0710**	**-2.00**	**down**
miR-16-5p	**0.0735**	**1.47**	**up**
miR-196b-5p	**0.0780**	**-2.04**	**down**
miR-17-5p	**0.0780**	**1.61**	**up**
miR-223-3p	**0.0829**	**4.02**	**up**
miR-7-5p	**0.0901**	**-2.66**	**down**
miR-520d-3p	0.0960	-2.27	down
miR-196a-5p	0.0961	-1.91	down
miR-376b-3p	0.1051	-3.33	down
miR-181d-5p	0.1071	-1.95	down
miR-130a-5p	**0.1086**	**-1.25**	**down**
miR-206	**0.1106**	**3.25**	**up**
miR-18a-5p	**0.1109**	**2.00**	**up**
miR-18b-5p	0.1123	1.97	up
let-7a-5p	0.1149	-1.31	down
miR-210-3p	**0.1168**	**1.64**	**up**
miR-9-5p	**0.1190**	**-2.45**	**down**
miR-135b-5p	0.1291	-2.95	down
miR-181a-2-3p	0.1325	-1.57	down
miR-199a-5p	**0.1363**	**-1.42**	**down**
miR-421	0.1388	1.54	up
miR-34c-5p	0.1448	-1.91	down
miR-155-5p	0.1535	1.92	up
miR-142-5p	0.1554	2.70	up
let-7g-5p	0.1557	1.28	up
miR-106a-5p	0.1596	1.52	up
miR-18b-3p	0.1633	-1.68	down
miR-519e-3p	0.1698	-1.75	down
miR-19b-3p	0.1712	1.57	up
miR-199b-5p	0.1729	-1.38	down

miR-125a-5p	0.1808	-1.48	down
miR-200a-3p	0.1817	2.00	up
miR-214-3p	0.1878	-1.64	down
miR-200b-3p	0.1953	-1.76	down
miR-181a-5p	0.1967	-1.87	down
miR-519a-3p	0.1999	-2.56	down

Cox proportional hazard univariable regression analysis of miRNA expression in 33 tumors from patients with DFI ranging from 20 to 937 days identified miRNAs associated with patient outcome (Table 3.5). The goal of multivariate Cox proportional hazard analysis in this study was to identify the best combination of candidate miRNAs whose expression explained a significant proportion of the variability of patient outcome in this group of tumors, and which would be likely to predict outcome in an independent set of canine tumors. Thus, expression values for seven miRNAs with $p<0.25$ based on the univariate analysis were included in both forward and backward step-wise multivariate Cox proportional hazard regression analysis. A three-miRNA model was selected as the best model based on Akaike information criterion (AIC), a measurement of model selection that takes into account goodness of fit of the model with penalties for increased complexity (Table 3.6).

Table 3.5: **Results of univariate Cox proportional hazard regression analysis for expression of miRNAs in canine OS tumors** (n=33, DFI range 20-937 days). Bold rows ($p<0.25$) selected for multivariate analysis.

miRNA Name	p-value	HR	95% CI
miR-223-3p	**0.00119**	**2.252**	**(1.379 - 3.68)**
miR-181b-5p	**0.0287**	**0.6481**	**(0.4394 - 0.9558)**
miR-130a-3p	**0.107**	**0.7022**	**(0.4567 - 1.08)**
miR-199a-5p	**0.158**	**0.7468**	**(0.4979 - 1.12)**
let-7b-5p	**0.171**	**0.6012**	**(0.29 - 1.246)**
miR-451a	**0.194**	**1.238**	**(0.8971 - 1.71)**

miR-7-5p	**0.236**	**1.22**	**(0.878 - 1.694)**
miR-26a-5p	0.315	0.8008	(0.5193 - 1.235)
miR-30c-5p	0.369	0.8559	(0.6095 - 1.202)
miR-142-3p	0.423	1.192	(0.776 - 1.831)
miR-206	0.583	0.9106	(0.6517 - 1.272)
miR-18a-5p	0.617	1.097	(0.7629 - 1.578)
miR-16-5p	0.648	0.9261	(0.666 - 1.288)
miR-196b-5p	0.668	0.92	(0.6282 - 1.347)
miR-9-5p	0.742	0.9403	(0.6517 - 1.357)
miR-135a-5p	0.788	0.9568	(0.6929 - 1.321)
miR-128-3p	0.796	0.9585	(0.6955 - 1.321)
miR-210-3p	0.964	1.009	(0.6953 - 1.463)
miR-17-5p	0.981	0.9962	(0.7327 - 1.354)

Table 3.6: **Three miRNA model with lowest AIC via both forward and backward step-wise Cox proportional hazard regression** (R^2=0.413, concordance = 0.73).

miRNA Name	**p-value**	**HR**	**95% CI**
miR-223-3p	0.000306	2.676	(1.5682 - 4.5663)
miR-130a-3p	0.022936	0.5718	(0.3532 - 0.9256)
let-7b-5p	0.145073	0.6034	(0.3059 - 1.1904)

Three-miRNA signature for patient outcome (DFI)

The Cox proportional hazard multivariate model with thee miRNAs, miR-223-3p, miR-130a-3p and let-7b-5p was used to calculate risk scores for each sample. The median risk score was used as a cut-off discriminating samples considered high or low risk. Kaplan-Meier survival analysis with the log rank test using the three-miRNA model based risk score distinguished patients with high risk and low risk with respective median DFIs of 123.5 days and 392 days

respectively (Figure 3.1A, p=0.0002, hazard ratio 3.2, 95% confidence interval 2.5-12.9). Additionally, if samples were separated into cohorts of good and poor responders based on mean DFI, the three-miRNA model signature had accuracy, based on area under the curve, of 0.86 (Figure 3.1B).

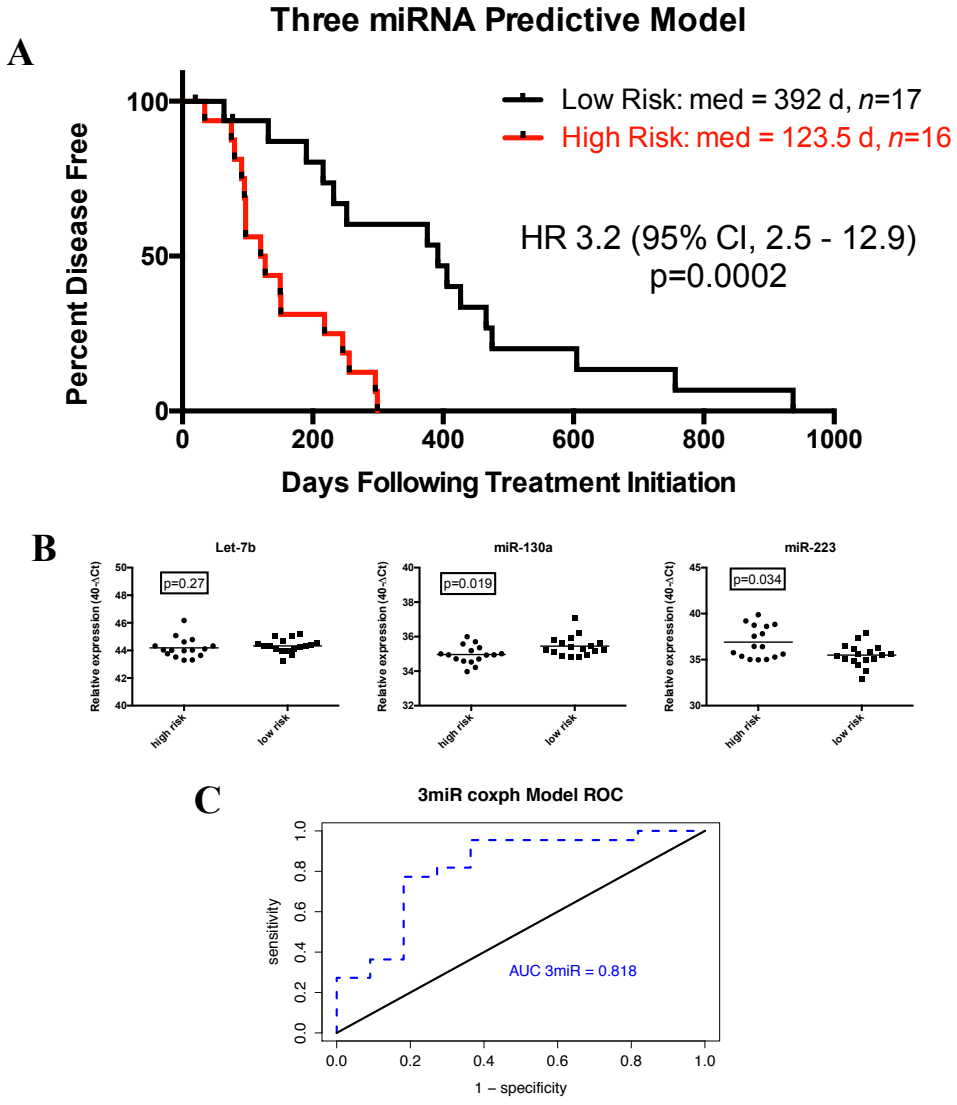

Figure 3.1: Three miRNA predictive Model. A. Kaplan Meier survival curve with log rank test (cut off is median risk score: 0.8897). B. Relative expression (40-ΔCt) of individual miRNAs in low and high risk groups (Mann Whitney test). C. Receiver operator characteristic curve for three-miR Cox proportional hazard based risk score dividing outcome groups based on mean DFI.

Pathway analysis of dysregulated miRNAs and genes suggest roles for tumor microenvironment and IGF2BP1 regulatory network in aggressive OS.

We used the mirPath tool from the Diana Tools website[20] to identify the top pathways enriched for genes that are targets of the three miRNAs in our cox proportional hazards model. The top 20 pathways include purine and pyrimidine metabolism, cell cycle, several cancers, and insulin signaling (Table 3.7). Interestingly, all of these pathways were based on targets of only let-7b and miR-223. The let-7 family, being among the earliest miRNAs discovered and more widely studied, shows 480 targets in this tool, while miR-223 and miR-130a list only 19 and 18 genes respectively. We repeated the mirPath query with only miR-223 and miR-130a to identify potential pathways involving these two miRNAs specifically (Table 3.8). In addition to several cancer-associated pathways, only three pathways showed involvement of genes targeted by both, Rheumatoid arthritis, osteoclast differentiation and hematopoietic cell lineage. This suggests dysregulaton of immune modulatory responses may facilitate aggressive OS.

Table 3.7: **Top 20 pathways enriched for genes targeted by miRNAs in the three-miRNA coxph model.**

KEGG pathway	p-value	#genes	#miRNAs
Ribosome biogenesis in eukaryotes	1.63E-07	11	1
Pyrimidine metabolism	1.13E-06	13	1
RNA transport	2.80E-06	15	1
Non-small cell lung cancer	3.25E-06	7	2
RNA polymerase	8.87E-06	6	1
Cell cycle	2.20E-05	11	2
Purine metabolism	8.13E-05	15	1
Chronic myeloid leukemia	0.000124855	8	2
Ubiquitin mediated proteolysis	0.000124958	12	1
Bladder cancer	0.000142215	6	2
Melanoma	0.000195154	7	2
Thyroid cancer	0.00019972	4	1
Selenocompound metabolism	0.000239832	4	1
Acute myeloid leukemia	0.000239832	6	2
Arrhythmogenic right ventricular cardiomyopathy	0.000281894	5	1

Glioma	0.000301037	7	2
Cytosolic DNA-sensing pathway	0.001057212	6	2
Prostate cancer	0.001174383	8	2
Insulin signaling pathway	0.002278599	10	1
Endometrial cancer	0.004156137	5	1

Table 3.8: **Top pathways (p<0.05) enriched for genes targeted by only miR-223 and/or miR-130a.**

KEGG pathway	p-value	#genes	#miRNAs
Rheumatoid arthritis	**0.001399064**	**2**	**2**
Cytosolic DNA-sensing pathway	0.001506064	2	1
Toll-like receptor signaling pathway	0.009601538	2	1
NOD-like receptor signaling pathway	0.009601538	2	1
Intestinal immune network for IgA production	0.009601538	1	1
Hepatitis B	0.009601538	3	1
Transcriptional misregulation in cancer	0.009601538	3	1
Chronic myeloid leukemia	0.0113375	2	1
Osteoclast differentiation	**0.0113375**	**2**	**2**
Pancreatic cancer	0.01167206	2	1
Small cell lung cancer	0.01167206	2	1
Prostate cancer	0.01310848	2	1
HTLV-I infection	0.0147781	3	1
Hematopoietic cell lineage	**0.0150344**	**2**	**2**
Chagas disease (American trypanosomiasis)	0.02321019	2	1
MAPK signaling pathway	0.03041792	3	1

We next used multiMiR, a miRNA-target interaction R package and database out of the Theodorescu lab[21] to identify either experimentally validated or predicted miRNA-mRNA interactions based on data from this and previous studies in our laboratory (Table 3.9). MultiMiR predicted potential interactions between miR-223, over-expressed in tumors from dogs with shorter DFI, and both dystonin (DST) and catenin (cadherin-associated protein), Alpha 2 (CTNNA2). Both are adhesion proteins interacting with the cytoskeleton; a second implication for involvement with the tumor environment potentially playing a role in the aggressiveness of OS. An interaction between miR-17 and CXCL14 further supports disruption of inflammation-associated pathways. A validated interaction between let-7b and IGF2BP1 and predicted

interactions between 6 other under-expressed miRNAs and IGF2BP1 confirms miRNA expression changes likely play a role in the high expression of this gene in tumors from dogs with the shortest DFI. Relative expression of let-7b and IGF2BP1 in 8 OS tumors via RT-qPCR confirms a statistically significant inverse correlation suggestive that this interaction occurs in canine OS (Figure 3.2)

Table 3.9: **miRNA-mRNA interactions between dysregulated miRNAs and mRNAs in tumors from dogs with short DFI as determined by multiMiR *in silico* analysis**. Bold interactions are discussed in the text.

Upregulated miRNAs and Downregulated Genes		
miRNA.ID	**Target.Gene**	**Evidence**
hsa-miR-142-3p	KIAA1191	Validated
hsa-miR-17-5p	SEPT11	Validated
hsa-miR-142-3p	ARID5B	Predicted
hsa-miR-142-3p	JAZF1	Predicted
hsa-miR-142-3p	EBF1	Predicted
hsa-miR-17-5p	CXCL14	Predicted
hsa-miR-17-5p	JAZF1	Predicted
hsa-miR-17-5p	CAMK2N1	Predicted
hsa-miR-17-5p	CCDC73	Predicted
hsa-miR-17-5p	KIAA1191	Predicted
hsa-miR-17-5p	IPO9	Predicted
hsa-miR-223-3p	**DST**	**Predicted**
hsa-miR-223-3p	**CTNNA2**	**Predicted**
Downregulated miRNAs and Upregulated Genes		
miRNA.ID	**Target.Gene**	**Evidence**
hsa-let-7b-5p	**IGF2BP1**	**Validated**
hsa-miR-26a-5p	OLA1	Validated
hsa-let-7b-5p	**PDZRN4**	**Predicted**
hsa-let-7b-5p	**DSCAM**	**Predicted**
hsa-miR-130a-3p	GDA	Predicted
hsa-miR-130a-3p	**IGF2BP1**	**Predicted**
hsa-miR-135a-5p	GRIA4	Predicted
hsa-miR-135a-5p	IGF2BP1	Predicted
hsa-miR-181b-5p	PDE10A	Predicted
hsa-miR-181b-5p	GDA	Predicted
hsa-miR-181b-5p	RAB11FIP1	Predicted
hsa-miR-196b-5p	IGF2BP1	Predicted
hsa-miR-196b-5p	RANBP3L	Predicted

hsa-miR-196b-5p	DSCAM	Predicted
hsa-miR-199a-5p	GRIA4	Predicted
hsa-miR-199a-5p	IGF2BP1	Predicted
hsa-miR-26a-5p	GRIA4	Predicted
hsa-miR-26a-5p	IGF2BP1	Predicted
hsa-miR-9-5p	IGF2BP1	Predicted

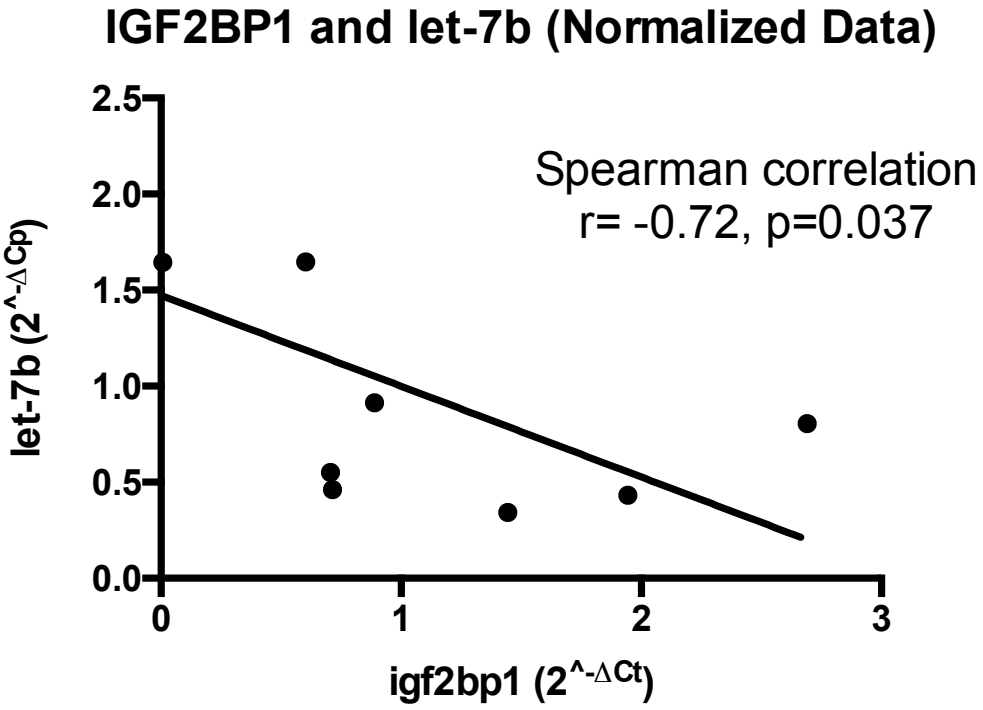

Figure 3.2. Correlation between low let-7b expression and high expression of IGF2BP1 in eight OS tumors as determined by RT-qPCR.

Differentially expressed miRNAs in OS tumors relative to normal bone support dysregulation of the Notch pathway in OS.

Expression of 188 miRNAs was also measured via RT-qPCR in 7 normal bone samples. As has been our experience with gene expression, more differentially expressed miRNAs were identified with higher statistical significance and larger fold changes. Forty differentially expressed miRNAs were identified using cut-offs of $p<0.05$ for significance and fold change greater than two; twenty-one miRNAs had lower expression in tumors than normal bone, while 19 miRNAs were over-expressed in tumors (Tables 3.10 and 3.11)

Table 3.10: **MicroRNAs up-regulated in tumors relative to normal bone.**

miR Name	Ttest P-val	Fold Change
miR-7-5p	2.5429E-08	24.58
miR-9-5p	0.0038	23.34
miR-346	0.0154	12.52
miR-96-5p	0.0001	8.11
miR-382-5p	0.0302	7.36
miR-663a	0.0000	7.08
miR-654-3p	0.0003	6.46
miR-493-3p	0.0132	5.75
miR-92b-3p	0.0027	5.19
miR-205-5p	0.0003	5.13
miR-18b-3p	0.0000	4.23
miR-135b-5p	0.0013	4.14
miR-132-3p	0.0005	4.01
miR-214-3p	0.0043	3.51
miR-199b-5p	0.0036	2.94
miR-331-5p	0.0347	2.50
miR-199a-5p	0.0256	2.29
miR-21-5p	0.0074	2.17
miR-328-3p	0.0012	2.08

Table 3.11: **MicroRNAs with lower expression in tumors compared to normal bone.**

miR Name	Ttest P-val	Fold Change
miR-107	3.7831E-08	-74.08
miR-133b	0.0068	-24.70
miR-141-3p	0.0012	-23.92
miR-206	0.0124	-20.20
miR-223-3p	0.0091	-18.72
miR-208-3p	0.0023	-17.97
miR-133a-3p	0.0231	-11.65
miR-150-5p	0.0002	-6.86
miR-146b-5p	0.0020	-5.11
miR-26a-5p	0.0025	-4.47
miR-29c-3p	0.0015	-3.64
miR-26b-5p	0.0000	-3.60
miR-129-5p	0.0310	-3.12
miR-34a-5p	0.0002	-2.94
miR-125b-5p	0.0065	-2.70
miR-29a-3p	0.0036	-2.60
miR-146a-5p	0.0010	-2.55

miR-30c-5p	0.0005	-2.52
miR-106b-5p	0.0066	-2.49
miR-99a-5p	0.0085	-2.29
miR-100-5p	0.0304	-2.09

Using multiMiR, we sought validated interactions between 21 downregulated miRs and 30 upregulated Notch/HES1-associated genes as well as between 19 upregulated miRs and 14 downregulated Notch/HES1-associated genes. The pool of Notch/HES1-associated genes was a subset of the genes evaluated in chapter 2. MultiMiR identified experimental, protein-based evidence for interactions between 22 of 41 miRs and 18 of 44 genes or roughly half of the miRNAs and genes entered into the analysis (Figure 3.2). This data supports the hypothesis, that dysregulation of the Notch signaling pathway contributes to the pathogenesis of OS and likely involves disruption of miRNA regulation of Notch pathway associated genes.

Serum miRNA changes associated with OS patient outcome

Expression of 13 miRNAs in 31 serum samples from patients with DFI ranging from 20 to 772 days was analyzed using a similar Cox proportional hazard regression pipeline described for tumor miRNA expression data. The thirteen miRNAs evaluated comprised a combination of 10 miRNAs selected from our analysis of tumor derived miRNA expression and three miRNAs commonly highly expressed in human serum samples. Forward and backward stepwise Cox multivariable proportional hazard regression analysis identified a two-miRNA model with the best fit based on AIC (Table 3.12). The risk score based on this model separated samples into groups with mean DFI of 272 days for the low risk group and 123.5 days for the high risk group (Figure 3.3 A, p=0.004, hazard ratio 2.6, 95% confidence interval 1.6 - 8.5)

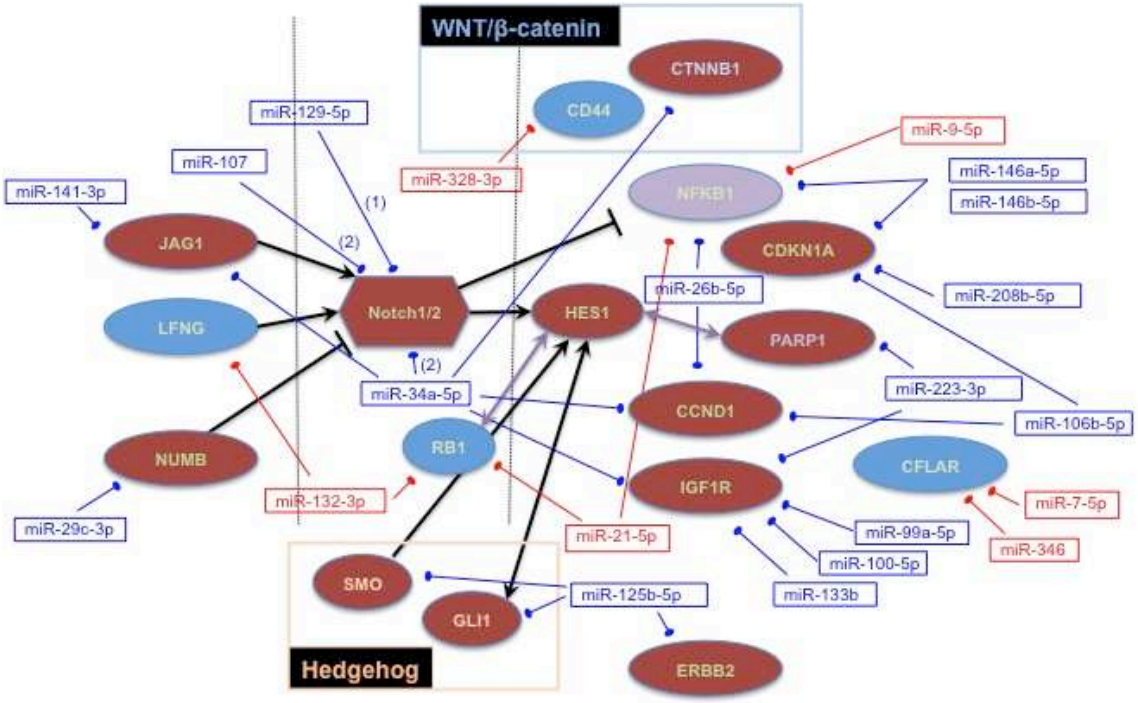

Figure 3.3. Notch/HES1 Associated miRNA-mRNA Interactions. Dysregulated genes are shown as ovals or polygons, dysregulated miRNAs are shown in text boxes. In both cases red indicates expression higher in tumors than normal bone, blue that expression is lower in tumors, and purple indicates that one probe in the Affymetrix array showed NFKB1 as upregulated and another as down regulated. Genes on the left are ligands or inhibitors of Notch; genes on the right are downstream targets of the Notch signaling pathway and/or specifically interact with HES1.

Table 3.12: **Two miRNA model after step-wise Cox proportional hazard regression** (R^2=0.278, concordance = 0.69).

miRNA Name	p-value	HR	95% CI
miR-23a	0.02091	0.5652	(0.3483 – 0.9173)
miR-30c-5p	0.00991	0.5487	(0.3477 – 0.8659)

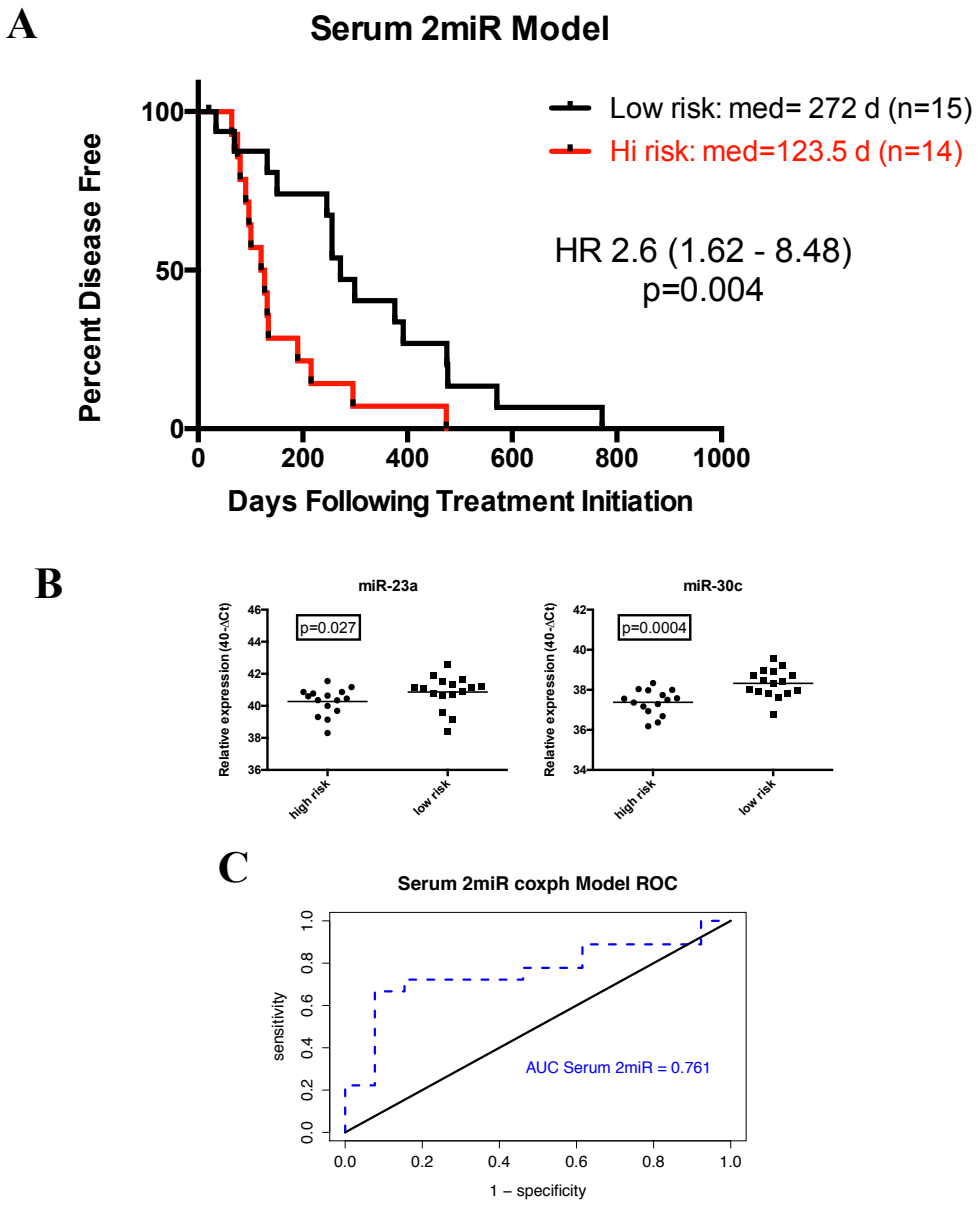

Figure 3.4. Serum 2 miRNA Model A. Kaplan Meier survival curve with log rank test (cut off is median risk score: 1.0372). B. Relative expression (40-ΔCt) of individual miRNAs in low and high risk groups (Mann Whitney test). C. Receiver operator characteristic curve for serum two-miR Cox proportional hazard based risk score dividing outcome groups based on mean DFI.

Tumor-based miRNA Signature Compared to Clinical Predictors

One measure of the value of a new prognostic biomarker is its usefulness compared to other predictive markers including clinical parameters.[22] For OS the most consistent clinical

indicators of outcome are proximal humerus location, weight and serum ALP.[1, 23-25] We had access to an expanded set of curated, quality checked clinical data for a subset of our tumors (n=24) that were included in a large retrospective study by Selmic et al.[23] Multivariate cox proportional hazard regression of the three miRNA expression based risk score and other clinical parameters ($p<0.25$ on univariate analysis) showed that when adjusting for these indicators, the miRNA expression based risk score remains a significant predictor of outcome (Table 3.13). This suggests that incorporation of miRNA expression signatures would improve estimation of prognosis for canine patients. Similar analyses were attempted with the 2miR serum-based risk score; however, only 12 patients in the Selmic group overlapped with our serum samples. This sample size was too small to adequately represent associations between clinical parameters and outcome.

Table 3.13: **Results of univariate/multivariate analysis of factors associated with clinical outcome including a three-miRNA expression based risk score** (tumor-derived miRNA expression).

		Univariate analysis			
		Med DFI (days)	**HR**	**P**	**95% CI**
3miR Risk Score	Low	392	0.18	0.00061	(0.0697 - 0.484)
	High	123.5			
Weight			1.05	0.046	(1.001 - 1.103)
Age at Dx			0.785	0.10	(0.587 - 1.051)
Proximal Humerus	Yes		3.055	0.057	(0.969 - 9.628)
	No				
		Multivariate analysis			
			HR	**P**	**95% CI**
3miR Risk Score			0.185	0.0067	(0.0546 - 0.626)
Proximal Humerus			5.63	0.016	(1.375- 23.06)

DISCUSSION

Aberrant miRNA expression patterns have been associated with patient outcome for a variety of human tumors. Combined with their stability in fixed tissues and less-invasively obtained body fluids, miRNAs make attractive candidates for biomarker discovery efforts. In this study we identified miRNA expression signatures from both canine OS tumor and patient serum samples that associated significantly with outcome following surgical amputation of the affected limb and standard of care chemotherapy. Pathway and miRNA-gene interaction analyses focused on tumor-derived miRNAs associated with poor outcome, suggested that interaction between OS cells and the primary tumor microenvironment may be a major determinant in the ultimate metastatic capabilities of OS tumor cells. Additional miRNA-gene interaction analyses combining expression changes identified in this study with gene expression changes from earlier studies suggest miRNA dysregulation contributes to both 1) disruption of the Notch pathway in OS compared to normal bone and 2) deregulation of the growth-promoting oncofetal protein IGF2BP1 in the most aggressive OS tumors. Finally, we demonstrated that the tumor-based three-miRNA signature remains an independent predictor of outcome when we control for possible effects of other clinical parameters such as tumor location, patient weight and age at diagnosis.

Our first goal of this study was to identify miRNAs associated with progression of OS despite standard of care treatment including surgical amputation and doxorubicin and/or platinum-based chemotherapy. We identified a three-miRNA expression signature that separated patients into two distinct outcome groups. Within this signature, elevated expression of miR-223, and decreased expression of let-7b and miR-130a were associated with increased risk and

ultimately shorter median DFI. Of these three, miR-223 was the most significantly associated with DFI based on p-value in both the univariate and multivariate regression analyses. Interestingly, expression of miR-223 is nearly 20 times lower in OS tumors compared to normal bone (table 3.11), consistent with two reports in human OS.[13, 26] miRNA expression analyses performed in canine cancer cell lines for experiments in Chapter 4 revealed miR-223 expression is uniformly low across canine osteosarcoma cell lines (data not shown). We performed pathway and miRNA-gene regulatory analyses to identify pathways potentially affected by expression changes in miR-223. These analyses suggest the significant increase in miR-223 contributing to short DFI in canine osteosarcoma tumors may be either originating from, or influenced by interactions with, the tumor microenvironment.

For example, pathways enriched for both miR-223 and miR-130a included hematopoetic cell development and osteoclast differentiation. Several lines of evidence support a role for miR-223 as an important regulator of the immune response inhibiting differentiation of classically activated (M1) macrophages and promoting the anti-inflammatory (M2) polarization.[27-29] Interestingly, Notch signaling is also important for classical pro-inflammatory M1 polarization.[30] Our findings of disrupted Notch signaling in aggressive OS in Chapter 1 could support potential inhibition of M1 polarization and/or promotion of M2 polarization of tumor associated macrophages in the most resistant OS tumors. MiR-223 is also important for normal differentiation and function of osteoclasts, also derived from bone marrow monocyte precursors.[31] Given that miR-223 is highly expressed by both M2 macrophages and osteoclasts, the possibility exists that the increased expression of miR-223 in tumors from poor responders is not coming from tumor cells, but from other cells in the tumor microenvironment. A second possibility is that expression of miR-223 in tumor cells might be induced by

association or interaction with increased numbers of these cells in the tumor microenvironment. For example, Yang et all demonstrated that M2 polarized macrophages can shuttle miR-223 via exosomal transport to breast cancer cells increasing their invasive ability.[37]

The role of both osteoclasts and macrophages in OS remains controversial due to a variety of factors including potentially different behaviors of these cells depending on level of differentiation, polarization and response to external stimuli.[32-34] Despite this uncertainty, macrophage activating agents, such as muramyl tripeptide phosphatidylethanolamine (L-MTP-PE), which activate macrophages towards the classical pro-inflammatory polarization (and thus inhibit M2 polarization) have consistently shown promise for treatment of OS.[35, 36]

Altered macrophage polarization or osteoclast differentiation may contribute to the aggressive canine OS phenotype, but our previous gene studies did not identify any of the direct targets of miR-223 thus far associated with these pathways. Our miRNA-gene interaction analysis did identify potential interactions between miR-223 and adhesion proteins DST and CTNNA2. Both are involved in actin cytoskeletal remodeling, a pathway commonly associated with metastasis and identified as enriched for dysregulated genes in aggressive OS tumors from our previous gene expression studies.[2] Changes in actin cytoskeletal remodeling, commonly triggered by cell-cell interactions also suggests a role for the tumor microenvironment in aggressive OS. Decreased expression of CTNNA2 in tumors from our poor responders, supports a pro-metastatic role for miR-223 as CTNNA2 acts as a tumor suppressor in both endometrial and laryngeal carcinomas.[41, 42] Additional evidence for an association between miR-223 and metastasis has been demonstrated in recurrent ovarian tumors and renal cell metastases.[38, 39] A recent report demonstrated the contribution of miR-223 to cisplatin resistance in gastric cancer cells.[40]

In contrast, two recent reports support a potential tumor suppressor role for miR-223 *in vitro* and one demonstrates an association between low miR-223 expression combined with elevated expression of its target gene, epithelial cell transforming sequence 2 (ECT2), and poor outcome based on expression in OS tissues.[27-29] It is worth noting that the only one of these studies using patient tissues included a mix of pediatric and adult tumors (age range 8 – 66 years).[28] Canine osteosarcoma most closely resembles the pediatric disease, while human adult OS is frequently associated with Paget's disease and may thus involve different underlying molecular mechanisms of progression.[1, 30]

Another goal of this study was to integrate miRNA and gene expression data changes identified in tumors from dogs with poor outcome compared to those with good outcome and in tumor compared to normal bone. We hoped to supplement our previously obtained gene expression data and identify key aberrant pathways contributing to pathogenesis and progression of OS. We have already identified a potential oncogenic interaction between elevated miR-223 expression and low CTNNA2 expression. Similar analysis focusing on under-expressed miRNAs and overexpressed genes revealed seven miRNAs with low expression in aggressive tumors and IGF2BP1, an oncogene of interest to our lab. IGF2BP1 has a 3' UTR that is thousands of kilobases long with numerous predicted and well-conserved binding sites for various miRNAs. This extended 3' UTR contains multiple polyadenylation sites, a mechanism by which the gene may avoid miRNA regulation.[3] We have confirmed that in tumors and several OS cell lines, the shorter 3' UTR predominates (unpublished data). We also found a statistically significant correlation between let-7b and IGF2BP1 via RT-qPCR in eight OS tumors for which we had measured both (figure 3.2). Identification of potential miRNA regulators of this protein will facilitate additional functional studies. Despite challenges with

effective delivery to target tissues, restoration of tumor suppressor miRNAs remains a rapidly growing area of research and studies such as this may identify new therapeutic miRNAs.

We did not identify any potential interactions between our most dysregulated miRNAs and HES1, nor did Notch signaling pop up in our pathway analyses focused on miRNAs aberrantly expressed between our DFI cohort tumors. This is consistent with our findings and those of Poos et al that Notch activation likely contributes to the proliferative response, but does not appear to drive metastasis.[43] To further explore the role of miRNAs in Notch activation in OS, we utilized miRNA expression changes identified by comparing tumors to normal bone. There is experimental evidence for interactions between nearly half of the miRNAs we entered into the analysis and one or one or more Notch/HES1 associated genes according to databases used in the multiMiR package. A handful of these pathways are targetable via small molecule inhibitors including Notch, Hedgehog, HER2/ERRB and PARP. Several of these have been or are under investigation for potential use in treatment of OS.[44] [45] Expression studies like ours might identify good biomarkers to help stratify patients into those most likely to respond to a given treatment or even identify biomarkers for monitoring therapeutic response.

The last aim of this study was to identify expression changes of presumed tumor-associated miRNAs in the serum associated with patient outcome. Reliable, repeatable RT-qPCR results for measurement of serum are challenging given that the amount of miRNA extracted from cell poor fluids such as serum or plasma can not be quantified or quality checked via typical methods. Thus, reactions are carried out based on sample volume and not RNA (cDNA) quantity. The addition of one or more synthetic miRNA from a different species and of known quantity can be added before extraction, measured during the PCR reaction, and used to help with identification of poor quality samples and data normalization. However, considerable

inconsistencies between circulating biomarker studies remains a considerable roadblock to the clinical utility and reliability of such screens.[46] This issue is not unique to circulating miRNAs, but plagues miRNA expression studies in general. [47, 48]

Despite these challenges, an underlying goal of this study was to show that we could carry out biomarker discovery efforts using a platform that was both affordable and practical in the veterinary setting. Using a similar platform to what we ultimately envision could be used in a clinical setting increases the translational potential of any proposed diagnostic screen as we move into additional validation and, ultimately, clinical trial scenarios. Thus we set out to identify a data analysis pipeline utilizing the same, relatively affordable, SYBR green RT-qPCR platform to measure relative expression of serum miRNAs. We were able ultimately to identify a two-miRNA signature which successfully stratified patients into distinct outcome groups. The most significantly altered miRNA in this signature was miR-30c which shows progressive decreased expression from normal bone to tumors (table 3.11) and from tumors from dogs with good outcome to tumors from dogs with poor outcome (table 3.4). While promising, it should be noted that for all of our miRNA-signatures predictive capability in an independent tumor set remains to be established.

In conclusion, we successfully identified miRNA expression changes associated with patient outcome in both OS tumor and patient serum samples. MicroRNA-gene interactions of the disrupted miRs in tumors with genes identified as aberrantly expressed by previous studies 1) suggested a “pro-metastatic niche” effect in the primary tumor microenvironment of tumors associates with decreased DFI, 2) confirmed altered regulation of IGF2BP1 in aggressive OS and Notch alterations in tumors compared to normal bone, and 3) identified targetable pathways

disrupted in OS. These studies support the value of miRNA expression studies in biomarker/target discovery efforts for OS.

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Chapter 4

Utilization of miRNA expression in OS cancer cells to identify drug-resistance-related microRNAs associated with outcome in canine tumors

SUMMARY

Osteosarcoma (OS) is the most common primary tumor of bone affecting approximately 10,000 dogs and 400 children or adolescents each year. Despite initial improvement in patient outcome in both species with the addition of chemotherapy, survival rates have plateaued in the last 20-30 years. As many as 80% of patients are thought to have clinically undetectable metastases at the time of presentation and thus, resistance to cytotoxic chemotherapies comprising standard of care treatment for OS likely plays a major role in progression of this disease.

Genomic based mutational and expression studies have identified associations and functional mechanisms contributing to resistance to a variety of conventional and targeted agents utilizing cell models of human cancers. Much of this work has relied on the NCI-60 panel of human tumor cell lines for which there is extensive curated molecular characterization data and drug sensitivity data for 119 approved anti-cancer drugs. Recent research efforts have focused on developing predictive algorithms incorporating drug sensitivity data in cancer cells to direct optimal treatment of cancer patients in an individualized medicine setting. Success of clinical implementation of such algorithms will rely on molecular biomarkers for characterization of tumors, stratification of patients and monitoring patient response.

MicroRNAs (miRNAs) are small non-coding RNA molecules involved in post-transcriptional gene regulation essential during development and to maintain homeostasis in adult tissues. MicroRNA dysregulation contributes to a variety of disease states including cancer as well as to cellular processes that confer resistance to anti-cancer drugs. We measured expression of over 2,000 mature miRNAs in 29 canine cancer cells for which drug sensitivity data for chemotherapy drugs, including doxorubicin and carboplatin, commonly used in veterinary medicine had been previously generated. We integrated information from our canine cells with similar publicly available data for the NCI-60 human cancer cells using algorithms similar to those reported for the co-expression extrapolation (COXEN) method to identify drug-resistance-related miRNAs for further evaluation in 33 canine OS tumors.

We identified miR-98, as a miRNA potentially associated with doxorubicin and/or-carboplatin resistance and participating in miRNA signatures associated with patient outcome. One implementation of the COXEN method integrating miRNA expression from microarray and RT-qPCR data failed to accurately predict doxorubicin sensitive vs. resistant tumors based on patient outcome, but provided encouraging results for future development of miRNA-based COXEN-type algorithms. Additionally, we identified potential interactions between miR-98, let-7b and miR-181b and dysregulated genes implicated in COXEN models for doxorubicin and carboplatin resistance in canine OS developed by the Gustafson laboratory. Pathway analysis of these genes implicated a role for the Notch signaling pathway in doxorubicin resistance. Finally, integrating data from Chapter 3 with findings here implicated the PI3K/mTOR pathway in progression of OS. In addition to identification of integrated pathways associated with progression and chemoresistance of OS, dysregulated miRNAs identified in this study may provide valuable biomarkers for direction and monitoring of treatment of OS.

INTRODUCTION

In Chapter Three we identified miRNAs, including a three-miRNA signature, associated with poor patient outcome for canine OS. Predictive signatures for prognosis may be useful clinically when counseling owners and making difficult decisions about treatment for OS. These signatures become even more valuable if they can be used to identify new targets or to predict response to a given treatment, thus directly influencing selection of optimal chemotherapy and/or targeted agents. This is in line with the goal of individualized medicine.

Given the role of miRNAs in regulating expression of genes involved in the balance of cell growth and cell death during development and in homeostasis of adult tissues, it is not surprising that their dysregulation has been associated with drug resistance. A growing body of literature, including comprehensive reviews, continues to demonstrate associations between miRNA expression changes and drug sensitivity as well as identifying targets and functional mechanisms involved.[1, 2] MiRNAs may inhibit apoptosis, promote angiogenesis, and alter the expression of receptors that are therapeutic targets or regulate other proteins known to contribute to drug resistance or to promote metastasis.[2] These discoveries have led to an expansion of efforts to use genomic data including gene and miRNA expression profiling in predictive modeling algorithms designed to predict sensitivity to therapies irrespective of tumor histiotype and to apply this knowledge in the clinic.[3]

One example of predictive modeling that was developed by the Theodorescu laboratory is an algorithm built around Co-expression extrapolation analysis (COXEN).[4] With this algorithm, Theodorecu's group utilized gene expression patterns associated with NCI-60 drug response data to predict response of independent cancer cells or patient tumors to a given

therapeutic.[4, 5] One benefit of the COXEN algorithm is that the set of cancer cells used to generate the drug resistance associated differential expression data does not have to include cancer cells from the same type of cancer as the tumors. Jared Fowles in the Gustafson laboratory at Colorado State University Flint Animal Cancer Center (CSU FACC), was able to successfully implement the COXEN pipeline based on gene expression in canine or human cancer cells to predict whether a given canine tumor would be sensitive or resistant to doxorubicin or carboplatin (unpublished data, personal communication).

The first aim of studies conducted in Chapter 4 was to measure miRNA expression in 29 canine cancer cells (FACC29) for which the CSU FACC (Jared Fowles) had previously established drug sensitivity data to commonly used chemotherapy drugs in canine oncology. Additionally, we collected miRNA expression data for the human NCI-60 cancer panel for which drug sensitivity data for 119 approved compounds is publically available. Using these datasets and the DFI tumor dataset from Chapter 3, we applied a COXEN-type approach to identify drug-resistance-associated miRNAs that might also be associated with patient outcome. In addition to the 19 miRNAs measured via RT-qPCR in our COS33 tumors, we applied variations of the COXEN pipeline to identify other miRNAs that were frequently incorporated in predictive models based on the cell line data. We selected 20 candidate miRNAs and successfully obtained RT-qPCR-based miRNA expression for 19 additional miRNAs in the COS33 tumors. Using these data sets, we developed and performed a modified COXEN analysis to attempt to predict sensitivity of 24 patient tumors to treatment with doxorubicin.

METHODS

Total RNA Isolation, Quantification, and Quality Assessment

Tumor RNA utilized for these experiments was the same as that prepared for Chapter 3 extracted via freeze-fracturing, TRIzol® Reagent (Life Technologies, Grand Island, NY) and purification with the mirVana™ miRNA extraction kit (Life Technologies, Grand Island, NY). After resuspension of the extracted RNA pellet in nuclease free water, the mirVana™ miRNA extraction kit (Life Technologies, Grand Island, NY) was used for additional RNA purification. For canine cells, extraction was performed using the mirVana miRNA™ extraction kit following the manufacturer's instructions. Lysis of cells from 10 cm plates was conducted when cells had reached >70% confluence. Cell culture conditions were as described in Chapter One. Following mirVana™ purification, DNAse treatment of RNA and assessment of purity, quality and quantity of both tumor and cell-derived RNA was performed as described in Chapter3.

MicroRNA Expression in Canine Cancer Cells

Purified and DNase treated RNA isolated from 29 canine cancer cells representing 11 different tumor types was sent to the Genomics and Microarray Core Facility at the University of Colorado Denver for array analysis with the GeneChip miRNA 4.0 Array (Affymetrix, Santa Clara, CA, USA).

Real-Time Reverse Transcriptase Quantitative PCR

cDNA synthesis of small non-coding RNAs was performed using the miScript II Reverse Transcription kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly, reverse transcription (RT) was performed in 10 µl reactions containing 500 ng of total RNA in 6

µl nuclease free water, 2 µl 5x HiSpec Buffer, 1 µl 10x Nucleics Mix and 1 µl Reverse Transcriptase Mix. Polyadenylation of miRNA and RT were carried out in parallel by incubating samples at 37°C for 60 minutes, followed by 95°C for 5 minutes (RT inactivation). Generated cDNAs were stored at -20°C until analysis.

Quantitative PCR measurements were performed in 384-well PCR plates in a 6 µl SYBR Green (Qiagen, Valencia, CA) reactions and the data was analyzed as described in Chapter 3. The same reference miRNAs identified in Chapter 3, miR-30a, miR-27b and miR-185, were used for data normalization.

Data Analysis

Microarray data was preprocessed using the Robust Multichip Average (RMA)[6] algorithm with $\log_2$ transformations. For initial explorations comparing resistant cells with sensitive cells for doxorubicin and OS cells with high migration phenotype to low migration phenotype, differential expression (DE) analysis was performed using the limma package in Bioconductor.[7] Limma was also used in the final COXEN model presented in this chapter. In all cases, a cut-off of significance was based on un-adjusted p-value <0.05. CIMminer was used to generate clustered images of the data as described in Chapter One. Migration status of OS cell lines was based on data from the Gustafson laboratory.

COXEN-like analysis for selection of candidate miRNAs for RT-qPCR analysis in 33 canine OS tumors: A modified version of the COXEN method for prediction model building was used to generate a list of promising miRNA biomarkers based on drug sensitivity for evaluation via RT-qPCR in 33 canine OS tumors. Differential gene expression analysis was performed using both limma and Significance Analysis of Microarrays (SAM) in both human and canine cell datasets. For the human data, we utilized publically available miRNA expression

data for human cells, specifically cancer cells from the NCI-60 panel, from the gene expression omnibus (GEO) database (GSE26375).[8] For the human miRNA expression data multiple probe sets were available for many genes. Data was first collapsed to the gene level using the *collapseRows* function from the R package, WCGNA,[9] by selecting one probe based on maximum variance between samples. DE was performed comparing resistant and sensitive cell lines based on GI50 data from the Developmental Therapeutics Program from the National Cancer Institute within the National Institutes of Health (http://dtp.nci.nih.gov). For canine samples DE was performed in the FACC29 array data comparing resistant and sensitive cell lines based on IC50s generated by Jared Fowles at CSU FACC. Drug sensitivity assays for select cell lines were replicated to confirm IC50 values.

The co-expression extrapolation (COXEN) step, which is designed to select a subset of miRNAs with strong concordant expression between two data sets, was performed using correlation matrices based on a protocol described by Lee et al[4]. Briefly correlations matrices were generated for differentially expressed miRNAs in either the human or canine cell dataset with matching miRNAs in the DFI tumor set from Chapter 3. Then a third matrix is created comparing rows of the matrixes created for the cell and tumor datasets to create a correlation value matrix. MiRNAs with correlation values greater than 90^{th} percentile were selected as being strongly co-expressed.

Finally prediction models for each pipeline were generated in Bioconductor using the Misclassification-Penalized Posteriors (MiPP) algorithm (Cho 2007, http://bioconductor.org/packages/release/bioc/html/MiPP.html). MiPP was run in human cancer cell, canine cancer cell, and canine OS tumor data for each set of "model genes" that were identified in the COXEN step with the following settings: sequential selection mode to n=5,

method =linear discriminant analysis (LDA), and splitting of the training set randomly to produce training and test sets within a single dataset. MiRNAs within the top 5 models in each dataset analyzed were collected into a large list of candidate miRNAs. All of these analyses were done for doxorubicin and carboplatin and a final set of 20 miRNAs were selected for RT-qPCR analysis in a larger set of canine OS samples.

COXEN analysis to predict sensitivity to doxorubicin in 24 OS tumors for which doxorubicin was part of the treatment: A COXEN analysis pipeline was followed as described above starting with differential analysis in the FACC29 canine cancer cell dataset. The identified differentially expressed miRNAs were tested for Co-expression against the 14 DFI tumors from Chapter three. MiPP was then performed with seven DFI tumors treated with doxorubicin as the training set and 23 tumors also treated with doxorubicin as the test set. COXEN scores of "sensitive" or "resistant" were used as class predictions to plot survival curves based on DFI and results of the Log Rank test were used to evaluate the accuracy of the predictions.

Cox proportional hazard regression analysis to identify predictive models based on RT-qPCR data in OS Tumors: Statistical analysis of survival data was performed as described in Chapter Three using a combination of the statistical package in Graphpad Prism and the survival and coxph packages in R. Associations between miRNA expression levels and DFI were evaluated using Cox proportional hazards linear regression. Multivariable Cox regression was then performed, utilizing both forward and backward stepwise models based on the Akaike information criterion (AIC). Variables with a univariate p-value of <0.25 were included in the multivariate analysis. Criteria for "best" Cox proportional multivariate models were as follows: 1) miRNAs included in the model had $p<0.25$ or lower in univariate analyses 2) the same models

were obtained with forward and backward stepwise regression 3) the number of miRNAs in the model was <15% of the number of samples (features) used to generate the model. The last criterion has been recommended to prevent over-fitting of the model, thus increasing the likelihood that the model would be predictive in an independent sample set. A risk score was calculated for each sample based on the best multivariate model and the Kaplan-Meier method was used to determine median DFI for low and high risk groups based on the median risk score. Comparison between groups was made with the log rank analysis and a p-value of <0.05 was considered significant.

RESULTS

*MicroRNA expression in canine cancer cells***.**

In order to identify miRNAs associated with resistance to a given drug, we first needed a dataset of miRNA expression in canine cancer cells. We extracted RNA from 29 canine cancer cells comprising the Colorado State University Flint Animal Cancer Center canine panel (FACC-29) and obtained miRNA expression data utilizing the Affymetrix miRNA4.0 microarray. This panel contains probes for over 30,000 mature microRNAs from 203 species including 2,578 mature human miRNAs and 292 canine miRNAs. Unbiased hierarchical clustering of the canine cell data using the top 100 most variable human miRNAs shows that cells tend to cluster together based on developmental lineage (Figure 4.1). This is consistent with findings of Lu et al who reported one of the earliest high throughput miRNA expression analysis studies using a variety of human tumor and cancer cell lines. [10]

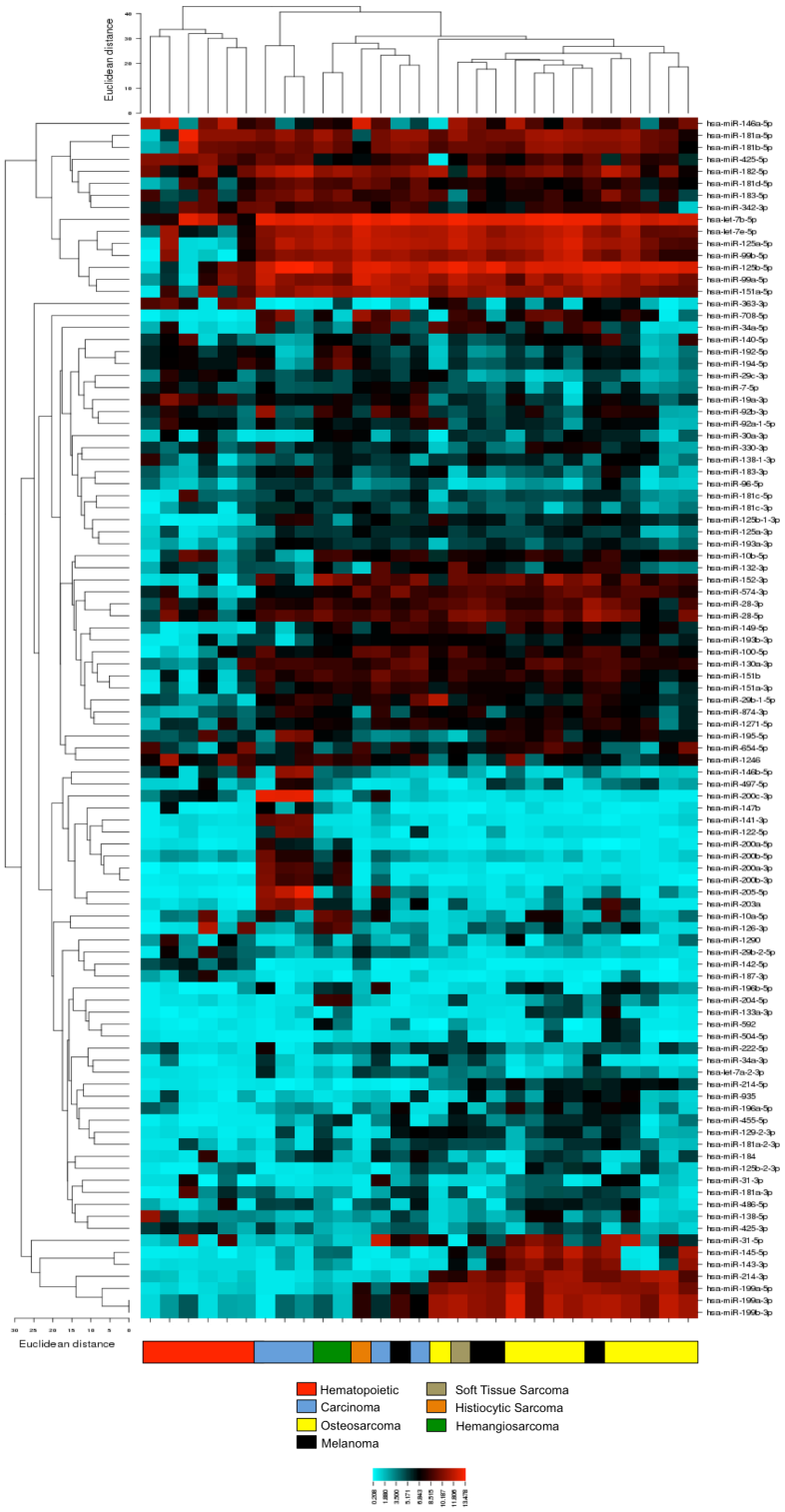

Figure 4.1. **Unbiased hierarchical clustering of canine cancer cells based on miRNA expression of top 100 most variably expressed miRNAs**. Cells of similar developmental lineage group together: hematopoetic cancer cells on the left (red), epithelial cancers in the middle (light blue), and sarcomas on the right (yellow, beige and black).

We chose to use the human probes for mature miRNAs in our analyses for several reasons. First, many of the miRNAs selected for evaluation in Chapter Three were selected based on analyses in human datasets or from information in the human literature. Some of the miRNAs we were interested in measuring do not have known canine counterparts yet. Given the high conservation of miRNAs observed in mammals, it is likely that these miRNAs have not yet been discovered and documented for dogs. Thus, using the human miRNA data might allow us to identify novel canine miRNAs associated with drug resistance and outcome. Secondly, many of our analyses depended on comparing miRNA expression between human and canine datasets. As the data we generated in Chapter Three and the human miRNA data in the Sokilde dataset all had human names, using human names and datasets would facilitate these comparisons. To demonstrate the similarity between miRNA expression data generated for human and canine miRNAs in canine OS cells we performed differential expression analyses for doxorubicin resistance cells compared to sensitive cells based on IC50 values and for OS cells with a high rate of migration via scratch assay compared to less migratory OS cells (Tables 4.1 and 4.2). In both cases 83.3 to 93.3% of canine miRNAs were identical to human miRNAs with similar fold changes, all in the same direction.

Table 4.1. **Differential expression of human and canine miRNAs in doxorubicin resistant canine cells compared to doxorubicin sensitive canine cells ($p<0.05$).** Values in bold are the same miRNAs with fold changes all in the same direction.

Human miRNAs			Canine miRNAs		
miR Name	**logFC**	**P.Value**	**miR Name**	**logFC**	**P.Value**
hsa-let-7f-5p	**1.24**	**0.0012**	**cfa-let-7f**	**1.24**	**0.0025**
hsa-let-7g-3p	**0.27**	**0.0437**	**cfa-let-7g**	**1.24**	**0.0015**
hsa-let-7g-5p	1.24	0.0006	**cfa-miR-103**	**0.81**	**0.0436**
hsa-let-7i-3p	0.75	0.0323	**cfa-miR-106a**	**1.49**	**0.0181**
hsa-let-7i-5p	1.55	0.0211	**cfa-miR-106b**	**1.41**	**0.0220**
hsa-miR-101-3p	1.06	0.0436	**cfa-miR-10a**	**4.05**	**0.0021**
hsa-miR-101-5p	0.57	0.0338	**cfa-miR-128**	**2.03**	**0.0227**
hsa-miR-103a-2-5p	1.80	0.0225	**cfa-miR-148b**	**2.30**	**0.0281**

hsa-miR-103a-3p	**0.81**	**0.0313**
hsa-miR-106a-5p	**1.47**	**0.0130**
hsa-miR-106b-3p	**0.78**	**0.0179**
hsa-miR-106b-5p	1.41	0.0189
hsa-miR-107	0.80	0.0330
hsa-miR-10a-5p	**4.88**	**0.0018**
hsa-miR-10b-3p	2.18	0.0398
hsa-miR-1180-3p	-0.64	0.0376
hsa-miR-1207-5p	-0.62	0.0085
hsa-miR-1225-3p	-0.42	0.0491
hsa-miR-1227-5p	-0.69	0.0324
hsa-miR-1228-3p	0.90	0.0073
hsa-miR-1228-5p	-0.86	0.0282
hsa-miR-1233-5p	-0.44	0.0320
hsa-miR-1246	2.55	0.0476
hsa-miR-1272	1.00	0.0065
hsa-miR-128-3p	**2.03**	**0.0215**
hsa-miR-1285-5p	-0.23	0.0477
hsa-miR-1287-3p	0.49	0.0127
hsa-miR-1303	-0.46	0.0111
hsa-miR-1306-5p	1.60	0.0103
hsa-miR-1307-5p	1.06	0.0086
hsa-miR-136-3p	-0.30	0.0274
hsa-miR-147a	0.35	0.0496
hsa-miR-148b-3p	**2.30**	**0.0273**
hsa-miR-152-5p	0.37	0.0282
hsa-miR-15a-3p	0.79	0.0354
hsa-miR-16-2-3p	1.08	0.0253
hsa-miR-17-3p	**2.90**	**0.0078**
hsa-miR-17-5p	1.48	0.0122
hsa-miR-186-5p	**2.11**	**0.0031**
hsa-miR-18a-3p	2.66	0.0217
hsa-miR-18a-5p	2.32	0.0117
hsa-miR-18b-5p	2.90	0.0215
hsa-miR-1908-5p	-0.53	0.0353
hsa-miR-1915-3p	-0.66	0.0479
hsa-miR-192-5p	**3.92**	**0.0017**
hsa-miR-194-5p	**3.96**	**0.0014**
hsa-miR-19a-3p	**3.55**	**0.0179**
hsa-miR-19b-3p	**2.04**	**0.0199**
hsa-miR-202-5p	0.79	0.0361
hsa-miR-20a-5p	**1.54**	**0.0158**
hsa-miR-20b-5p	2.00	0.0323
cfa-miR-15b	1.19	0.0463
cfa-miR-17	**2.90**	**0.0081**
cfa-miR-1841	0.97	0.0500
cfa-miR-1842	2.82	0.0154
cfa-miR-186	**2.11**	**0.0036**
cfa-miR-192	**3.92**	**0.0017**
cfa-miR-194	**3.96**	**0.0015**
cfa-miR-19a	**3.55**	**0.0179**
cfa-miR-19b	**2.00**	**0.0244**
cfa-miR-20a	**1.54**	**0.0182**
cfa-miR-25	**1.16**	**0.0330**
cfa-miR-26b	**2.83**	**0.0022**
cfa-miR-30b	**1.50**	**0.0028**
cfa-miR-30c	**1.37**	**0.0019**
cfa-miR-30e	**2.13**	**0.0094**
cfa-miR-342	**1.93**	**0.0422**
cfa-miR-421	2.48	0.0363
cfa-miR-450b	0.90	0.0273
cfa-miR-503	**1.37**	**0.0383**
cfa-miR-92b	**3.14**	**0.0152**
cfa-miR-93	**0.73**	**0.0340**
cfa-miR-98	**2.26**	**0.0013**

hsa-miR-211-5p	-0.30	0.0419
hsa-miR-25-3p	**1.16**	**0.0277**
hsa-miR-26b-5p	**2.70**	**0.0022**
hsa-miR-29b-2-5p	2.32	0.0288
hsa-miR-301a-3p	2.82	0.0231
hsa-miR-30b-5p	**1.50**	**0.0019**
hsa-miR-30c-5p	**1.47**	**0.0014**
hsa-miR-30d-5p	1.07	0.0034
hsa-miR-30e-3p	**2.13**	**0.0085**
hsa-miR-30e-5p	2.86	0.0076
hsa-miR-328-5p	-0.57	0.0090
hsa-miR-339-5p	0.86	0.0414
hsa-miR-33a-3p	0.38	0.0439
hsa-miR-342-3p	**1.93**	**0.0408**
hsa-miR-342-5p	1.27	0.0290
hsa-miR-378d	1.53	0.0498
hsa-miR-422a	1.44	0.0498
hsa-miR-494-3p	2.27	0.0176
hsa-miR-496	0.34	0.0123
hsa-miR-502-5p	2.04	0.0193
hsa-miR-503-5p	**1.85**	**0.0322**
hsa-miR-505-3p	2.00	0.0091
hsa-miR-505-5p	1.00	0.0348
hsa-miR-509-3-5p	0.36	0.0371
hsa-miR-509-3p	-0.27	0.0364
hsa-miR-512-3p	0.80	0.0481
hsa-miR-523-3p	-0.26	0.0254
hsa-miR-551b-3p	-0.29	0.0441
hsa-miR-619-5p	0.98	0.0287
hsa-miR-638	-0.68	0.0248
hsa-miR-758-5p	-0.41	0.0305
hsa-miR-769-5p	1.43	0.0365
hsa-miR-888-5p	-0.24	0.0362
hsa-miR-891a-3p	0.42	0.0164
hsa-miR-891b	0.33	0.0450
hsa-miR-92b-3p	**3.14**	**0.0150**
hsa-miR-93-3p	**1.26**	**0.0269**
hsa-miR-93-5p	0.73	0.0199
hsa-miR-940	0.92	0.0472
hsa-miR-98-5p	**2.26**	**0.0011**
hsa-miR-99b-3p	0.44	0.0315

Table 4.2. **Differential expression of human and canine miRNAs in OS cells with increased migration ability compared to OS cells with low migratory ability (p<0.05).** Values in bold are the same miRNAs with fold changes all in the same direction.

Human miRNAs		
miR Name	**logFC**	**P.Value**
hsa-miR-106a-5p	**-1.22**	**0.0281**
hsa-miR-1228-3p	-1.52	0.0134
hsa-miR-1237-3p	0.64	0.0326
hsa-miR-126-3p	-2.22	0.0321
hsa-miR-1272	-0.60	0.0379
hsa-miR-1283	0.50	0.0405
hsa-miR-129-5p	**2.00**	**0.0317**
hsa-miR-1306-3p	-0.99	0.0281
hsa-miR-1306-5p	-1.44	0.0144
hsa-miR-17-3p	**-2.65**	**0.0078**
hsa-miR-17-5p	-1.24	0.0239
hsa-miR-18a-5p	-1.90	0.0047
hsa-miR-18b-5p	-2.30	0.0123
hsa-miR-1910-5p	-1.32	0.0254
hsa-miR-195-5p	**-2.76**	**0.0179**
hsa-miR-196b-3p	**-2.03**	**0.0019**
hsa-miR-196b-5p	-4.14	0.0046
hsa-miR-19a-3p	**-3.15**	**0.0174**
hsa-miR-221-3p	**1.39**	**0.0052**
hsa-miR-221-5p	1.84	0.0489
hsa-miR-222-3p	1.38	0.0150
hsa-miR-222-5p	2.56	0.0417
hsa-miR-300	-0.73	0.0071
hsa-miR-323a-5p	1.16	0.0024
hsa-miR-328-5p	0.50	0.0103
hsa-miR-338-3p	-0.51	0.0304
hsa-miR-34b-3p	-0.39	0.0314
hsa-miR-362-5p	-1.63	0.0277
hsa-miR-365a-3p	-0.42	0.0499
hsa-miR-365b-3p	-0.42	0.0499
hsa-miR-494-3p	-1.89	0.0230
hsa-miR-497-5p	**-2.94**	**0.0185**
hsa-miR-500a-3p	**-1.42**	**0.0207**
hsa-miR-500a-5p	-1.53	0.0113
hsa-miR-501-3p	-2.47	0.0102
hsa-miR-502-3p	**-1.24**	**0.0081**
hsa-miR-505-3p	-1.79	0.0091

Canine miRNAs		
miR Name	**logFC**	**P.Value**
cfa-miR-106a	**-1.24**	**0.0406**
cfa-miR-129	**2.00**	**0.0354**
cfa-miR-17	**-2.65**	**0.0091**
cfa-miR-195	**-3.49**	**0.0267**
cfa-miR-196b	**-3.95**	**0.0031**
cfa-miR-19a	**-3.15**	**0.0185**
cfa-miR-221	**1.55**	**0.0120**
cfa-miR-497	**-2.94**	**0.0198**
cfa-miR-500	**-1.30**	**0.0191**
cfa-miR-502	**-1.24**	**0.0156**
cfa-miR-532	**-1.66**	**0.0216**
cfa-miR-660	**-1.98**	**0.0468**
cfa-miR-92b	**-3.41**	**0.0103**
cfa-miR-1839	-1.75	0.0272

hsa-miR-532-5p	**-1.66**	**0.0168**
hsa-miR-548d-5p	-0.52	0.0151
hsa-miR-548k	0.46	0.0400
hsa-miR-564	-0.87	0.0309
hsa-miR-567	-0.51	0.0197
hsa-miR-590-5p	-0.39	0.0420
hsa-miR-660-5p	**-1.98**	**0.0428**
hsa-miR-671-3p	-1.83	0.0186
hsa-miR-891b	-0.59	0.0124
hsa-miR-92b-3p	**-3.41**	**0.0096**

Unbiased hierarchical cluster analysis of the top differentially expressed miRNAs between OS cells with low and high migration phenotype ($p<0.05$, FC > 1.5) correctly groups the cells and shows the majority of differentially expressed miRNAs have lower expression in the more migratory cells. This is consistent with other studies and with an overall role for miRNAs providing tight regulation of cell processes involved in migration of cells during development. Interestingly, a handful of the differentially expressed miRNAs were among those investigated in Chapter 3 as associated with poor prognosis; though none made it into our final model. MiR-196b did show a trend toward lower expression in 15 tumors from canine patients with DFI<100 days compared to 14 tumors from dogs with DFI >300 days ($p=0.070$, FC=-1.66). This may suggest a role for miR-196b in OS cell migration contributing to metastasis and poor patient outcome.

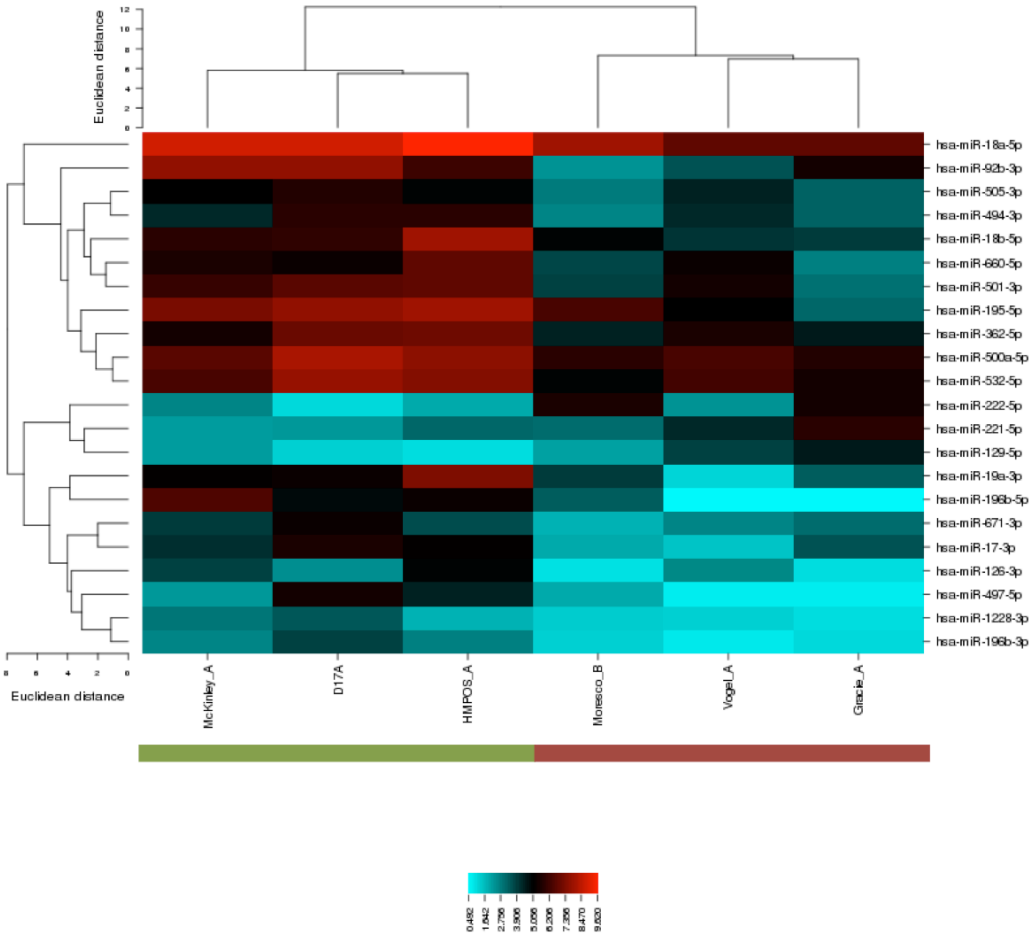

Figure 4.2. Unbiased hierarchical cluster analysis based on human miRNAs of OS cells with high migration rate vs. OS cells with low migration rate ($p<0.05$, FC>1.5). Red bar indicates cells with high migration rate, green bar cells with low migration rate.

MiRNA expression profiling of cancer cells based on chemotherapy sensitivity identify miR-98, associated with resistance to doxorubicin in canine cells, is also associated with patient outcome.

Utilizing expression data for human miRNAs measured in our canine samples, we next sought to identify candidate miRNAs associated with drug resistance as candidate biomarkers for poor outcome as a measure of poor response. Previous studies by Jared Fowles have identified cell-based models predictive of response to chemotherapy in canine tumors using co-expression extrapolation analysis (COXEN).[4] Briefly, this pipeline determines differentially expressed genes between a group of cancer cells sensitive to a given drug based on experimental data (IC50

or GI50) and a group of resistant cells. The so-called COXEN step utilizes correlation matrices to look for patterns of gene expression shared between the cell dataset and a reference set, typically a dataset of gene expression in the tumors of interest. The output of the COXEN step is a smaller, more refined set of candidate genes, which are input to additional modeling algorithms to build a model that will produce a "COXEN score" for each tumor correlating to a prediction that the tumor will be sensitive or resistant to the given drug.

We decided to implement the first part of the COXEN algorithm, starting with differential expression in either human (NCI60) or canine (FACC29) cells based on doxorubicin or carboplatin sensitivity data. Ultimately we selected and subsequently measured expression of 20 "drug-associated" miRNAs in 33 canine OS tumors with DFI ranging from 20 to 937. Data for one miRNA was dropped from further analysis based on low expression and amplification in "NoRT" negative controls. Univariate Cox proportional hazards regression analysis of expression of the 19 miRNAs showed none were significantly associated with outcome using a cut-off of $p<0.05$ (Table 4.3). A multivariate model with the top three miRNAs ($p<0.25$) from the univariate analysis failed to identify a model that would classify tumors into distinct outcome groups even after stepwise regression (data not shown).

Table 4.3. Univariate Cox proportional hazard regression analysis of 19 drug-associated miRNAs in 33 tumors treated with doxorubicin, carboplatin or a combination.

miRNA Name	p-value	HR	95% CI
hsa-miR-30d-5p	0.078	0.71	(0.48-1.04)
hsa-miR-181a-2-3p	0.170	0.77	(0.53-1.12)
hsa-miR-98-5p	0.243	1.22	(0.87-1.70)
hsa-miR-200c-3p	0.288	1.20	(0.85-1.70)
hsa-miR-194-5p	0.338	1.07	(0.71-1.62)
hsa-miR-141-3p	0.474	0.89	(0.63-1.24)
hsa-miR-301a-3p	0.478	1.13	(0.81-1.59)
hsa-miR-27a-3p	0.495	0.88	(0.60-1.28)
hsa-let-7g-5p	0.511	1.13	(0.79-1.62)

hsa-miR-92a-3p	0.515	1.19	(0.71-1.99)
hsa-miR-21-5p	0.517	1.12	(0.80-1.57)
hsa-miR-340-5p	0.566	1.12	(0.76-1.66)
hsa-let-7f-5p	0.595	1.11	(0.76-1.62)
hsa-miR-19a-3p	0.654	1.09	(0.75-1.59)
hsa-miR-20b-5p	0.683	0.93	(0.68-1.30)
hsa-miR-106a-5p	0.859	0.97	(0.73-1.47)
hsa-miR-141-3p	0.877	1.03	(0.70-1.51)
hsa-miR-125b-5p	0.886	1.03	(0.71-1.49)
hsa-miR-25-3p	0.950	0.99	(0.69-1.41)
hsa-miR-18b-5p	0.985	1.00	(0.72-1.38)

While we did not identify a good signature associated with outcome with only the drug-associated miRNAs, we used multivariate Cox proportional hazards stepwise regression to re-analyze the full set of 33 tumors with 10 of the 38 miRNAs for which we had measured expression and for which the univariate analysis resulted in a $p<0.25$. Thus we essentially added three of the drug-associated miRNAs into our previous multivariate analysis, miR-30d, miR-98 and miR-181a2. This resulted in a new "best model" which included the three miRNAs identified previously, let-7b, miR-130a, miR-223 with the addition of miR-98 from the drug-associated set of miRNAs (figure 4.4A). This model appears to separate tumors similarly to the 3-miRNA model with slightly improved separation immediately following treatment (Figure 4.3A and B).

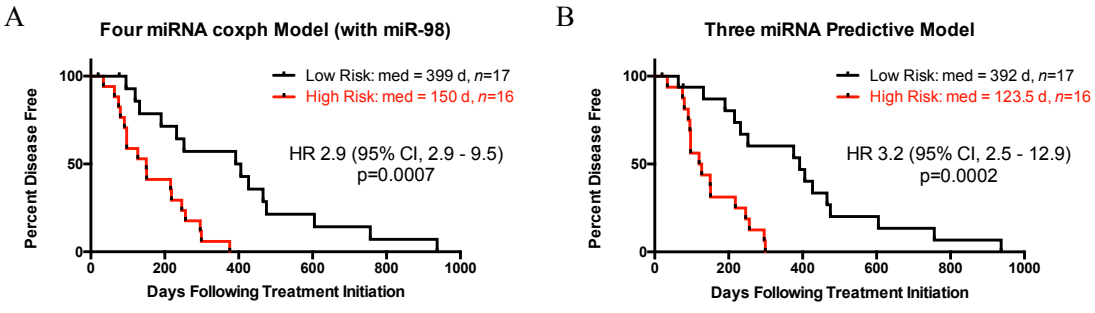

Figure 4.3. Four miRNA signature including miR-98 (A) compared to three miRNA signature discussed in Chapter 3 (B). Kaplan Meier analysis of high and low risk patients based on Cox proportional hazard models using median score as the cutoff and the log rank test.

Given that some of the drug-associated miRNAs were selected based on an association with sensitivity to either doxorubicin or carboplatin, it may not be appropriate to expect them to associate with outcome in the subset of tumors among the 33 tumors that were not treated with that drug. In other words, of these 33 tumors, 7 were treated with only carboplatin. If the miRNA under investigation had an association with doxorubicin resistance it may not have any association with outcome in tumors treated only with carboplatin. We did not have enough tumors treated with single agents to separate our samples into pure treatment groups, but we did repeat the Cox proportional hazard regression modeling pipeline in two subsets of our 33 tumors: a doxorubicin treated group (dox group) consisting of tumors treated with doxorubicin or a combination of drugs that included doxorubicin (n=23 tumors) and a similar group of carboplatin treated tumors (n=26, carbo group). Table 4.4 shows miRNAs that associate with DFI in the dox group based on $p<0.25$ in the univariate analysis and corresponding analysis for those miRNAs in the carbo group (carbo). There were no additional miRNAs with $p<0.25$ in the cox group that were not on this list. Tables 4.5 and 4.6 show results of multivariate Cox proportional hazard regression analysis with forward and backward stepwise regression based on AIC for each subset. The best model is shown based on criteria specified in the methods. Kaplan Meier survival curves with the log rank test for each model show that risk scores based on the Cox proportional multivariate models separate patients into groups with median DFI >300 days and median DFI 100-150 days (Figure 4.4 A and B).

Table 4.4. Results of univariate Cox proportional hazard analysis in treatment subgroups (top 12 based on analysis in dox group shown). HR=hazard ratio, CI=confidence interval

	Dox/Dox+Carbo (n=23)				Carbo/Dox+Carbo (n=26)		
miRNA Name	**p-value**	**HR**	**95% CI**		**p-value**	**HR**	**95% CI**
hsa-let-7b-5p	0.005	0.20	(0.067 - 0.62)		0.586	0.81	(0.38 - 1.71)
hsa-miR-181b-5p	0.017	0.57	(0.36 - 0.90)		0.086	0.69	(0.45 - 1.06)
hsa-miR-98-5p	0.042	2.57	(1.04 - 6.37)		0.210	1.24	(0.89 - 1.72)
hsa-miR-223-3p	0.054	1.84	(0.99 - 3.43)		0.005	2.123	(1.26 - 3.58)
hsa-miR-199a-5p	0.140	0.70	(0.43 - 1.13)		0.321	0.8069	(0.53 - 1.23)
hsa-miR-130a-3p	0.174	0.74	(0.48 - 1.14)		0.175	0.75	(0.50 - 1.14)
hsa-miR-196b-5p	0.191	0.72	(0.45 - 1.18)		0.752	0.93	(0.61 - 1.44)
hsa-miR-7-5p	0.205	1.24	(0.89 - 1.72)		0.296	1.20	(0.85 - 1.71)
hsa-miR-30c-5p	0.234	0.78	(0.52 - 1.17)		0.348	0.832	(0.57 - 1.22)
hsa-miR-30d-5p	0.238	0.75	(0.47 - 1.21)		0.105	0.72	(0.48 - 1.07)
hsa-miR-26a-5p	0.242	0.73	(0.42 - 1.24)		0.389	0.831	(0.55 - 1.27)
hsa-miR-301a-3p	0.249	1.34	(0.81 - 2.21)		0.619	1.093	(0.77 - 1.55)

Table 4.5. Multivariate Cox proportional analysis for tumors from doxorubicin-treated dogs. HR=hazard ratio, CI=confidence interval (Concordance = 0.828, R^2=0.62)

Best Doxorubicin Model

miRNA Name	**p-value**	**HR**	**95% CI**
hsa-let-7b-5p	0.0096	0.25	(0.09 -0.72)
hsa-miR-181-5p	0.015	0.50	(0.28 - 0.88)
hsa-miR-98-5p	0.0048	5.10	(1.64 - 15.80)

Table 4.6. **Multivariate Cox proportional analysis for tumors from carboplatin-treated dogs**. HR=hazard ratio, CI=confidence interval (Concordance = 0.735, R^2=0.41)

Best Carboplatin Model

miRNA Name	**p-value**	**HR**	**95% CI**
hsa-miR-130a-3p	0.073	0.66	(0.42-1.04)
hsa-miR-223-3p	0.00084	2.66	(1.50-4.73)
hsa-miR-98-5p	0.082	1.36	(0.96 -1.93)

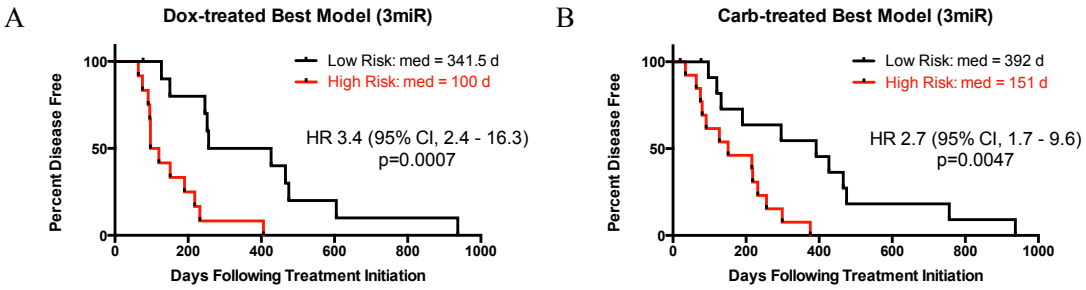

Figure 4.4 Survival curves for the best models in the doxorubicin and carboplatin treated subsets of OS tumors (Kaplan Meier method with log rank test).

These models may suggest different miRNAs are important for predicting outcome in doxorubicin treated dogs vs. carboplatin treated dogs; however, no definitive conclusions can be drawn as both subsets include tumors from dogs treated with both doxorubicin and carboplatin making up more than 50% of the tumors examined. Also, it should be noted that while the best models are shown, the original 3-miR signature and the 4-miR signature also successfully separate patients in the dox and carbo subgroups. These models simply demonstrate that, no matter how the data is grouped, five miRNAs continue to arise, either in univariate analyses or in multivariate models, as significantly associated with patient outcome: let-7b, miR-98, miR-130a, miR-181b, and miR-223.

Addition of miRNA-98 to analyses of up-regulated miRNAs and down-regulated genes in multiMiR reveals three predicted interactions including suppression of DST discussed in Chapter 3 (Table 4.7).

Table 4.7. Predicted interactions between miR-98 and dysregulated genes in tumors from dogs with poor response. DST=dystonin; LAMA1=lamanine; alpha 1, SNAP91=synaptosomal-associated protein, 91kDa

miRNA.ID	Target.Gene	Evidence
hsa-miR-98-5p	DST	Predicted
hsa-miR-98-5p	LAMA1	Predicted
hsa-miR-98-5p	SNAP91	Predicted

Finally, expanding pathway analyses performed in Diana Tools mirPath in Chapter Three to include miR-98 and miR-181b shows consistent enrichment many of the same pathways including cell cycle, pyrimidine and purine metabolism, insulin signaling and pathways contributing to various cancers. Interestingly, of the top 25 enriched pathways, only the PI3K-AKT pathway shows enrichment for genes targeted by all five miRNAs of interest (Figure 4.8).

Table 4.8. Top 25 pathways enriched for genes targeted by top five miRNAs of interest. MicroRNAs selected for association with outcome, association with resistance to chemotherapy, and potential predictive capabilities (Diana Tools mirPath).

KEGG pathway	p-value	#genes	#miRNAs
Viral carcinogenesis	4.03405E-11	34	3
RNA transport	1.09588E-06	22	2
RNA polymerase	1.09588E-06	8	2
Cell cycle	1.09588E-06	19	3
Insulin signaling pathway	5.68768E-06	19	2
Chronic myeloid leukemia	5.68768E-06	13	3
Ribosome biogenesis in eukaryotes	1.00676E-05	13	2
Bladder cancer	1.36754E-05	9	3
Small cell lung cancer	1.36754E-05	13	4
Pyrimidine metabolism	4.63135E-05	17	2
Prostate cancer	4.87094E-05	13	4
Hepatitis B	5.17479E-05	20	4
Purine metabolism	6.90832E-05	23	2
Acute myeloid leukemia	0.00021	9	3
Pathways in cancer	0.00021	30	4
Cytosolic DNA-sensing pathway	0.00024	9	3
HIF-1 signaling pathway	0.00037	15	4
Melanoma	0.00039	10	3

Non-small cell lung cancer	0.00053	8	3
NF-kappa B signaling pathway	0.00071	13	4
Systemic lupus erythematosus	0.00090	19	2
Thyroid cancer	0.00244	5	2
Epstein-Barr virus infection	0.00316	20	4
PI3K-Akt signaling pathway	**0.00375**	**30**	**5**
Alcoholism	0.00788	20	2

COXEN-related analyses suggest miRNA interactions contribute to dysregulation of genes perturbed in doxorubicin and carboplatin predictive COXEN models.

Twelve of the 38 miRNAs for which we had RT-qPCR expression data in our 33 canine OS tumors, were among 15 miRNAs identified as candidate model genes based on one pipeline of COXEN analysis. Differential expression in the FACC29 tumors based on doxorubicin sensitivity and COXEN with 175 miRNAs in 14 DFI tumors produced a list of 15 model genes. We used RT-qPCR expression data for 12 of those genes to build models in the seven DFI tumors from dogs whose treatment included doxorubicin and tested the top five models in 24/33 canine tumors in the test set whose treatment also included doxorubicin. The results of the top COXEN model for doxorubicin are shown in Figure 4.5. In general, all of the models generated from these 12 miRNAs tended to misclassify some resistant tumors as sensitive. In the best model this misclassification leads to a failure to appropriately categorize tumors at early time points. We attribute this high rate of misclassification as being secondary to limitations of our implementation of the COXEN algorithm, including crossing between microarray and RT=qPCR platforms and thus restricting the pool of potential model miRNAs to less than 200 miRNAs from our RT-qPCR experiment.

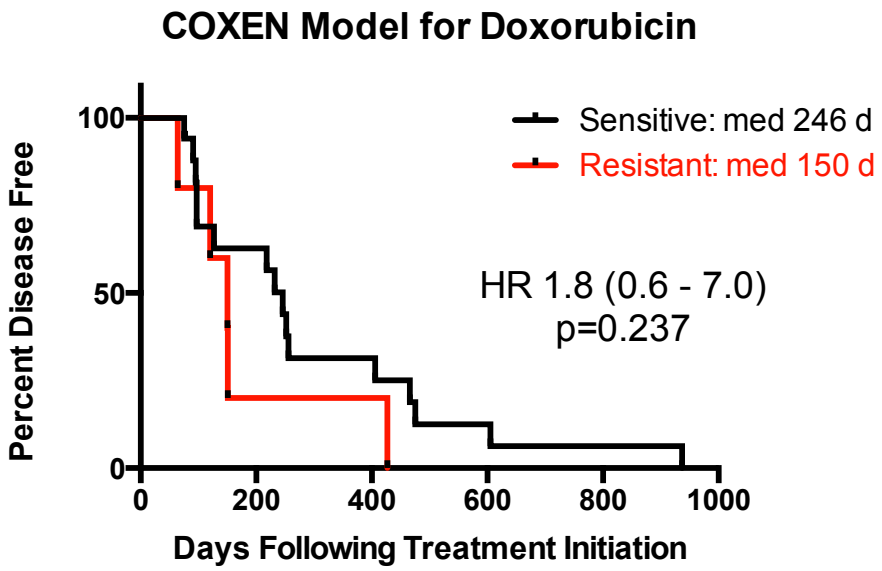

Figure 4.5. Doxorubicin COXEN scores for 24 OS tumors based on miRNA expression. Kaplan Meier method with log rank test.

Recently, Jared Fowles in the Gustafson laboratory at the CSU FACC identified highly predictive gene-expression based COXEN models for sensitivity to doxorubicin and carboplatin in canine OS. We utilized multiMiR to identify potential interactions between dysregulated miRNAs identified in Chapter Three and in analyses presented here and the genes from COXEN doxorubicin and carboplatin models (Table 4.9). Interactions between seven miRNAs, including three of our top 5 outcome associated miRNAs shown in bold, and four COXEN genes were identified. Notably, pathway analyses based on the four COXEN genes, using the publically available Enrichr pathway analysis tool[11], identified enrichment of the Notch signaling pathway (Table 4.10).

Table 4.9. Predicted interactions between dysregulated miRNAs and genes predictive of drug sensitivity in OS tumors. EIF6=eukaryotic translation initiation factor 6; TES=testin LIM domain protein; TLE1=transducine-like enhancer of split 1 (E(sp1) homolog, Drosophila

miRNA.ID	Target.Gene	Evidence	COXEN Model Drug
hsa-miR-98-5p	**KIAA0922**	**Validated**	**Carboplatin**
hsa-miR-98-5p	**TES**	**Validated**	**Doxorubicin**
hsa-let-7b-5p	**TES**	**Validated**	**Doxorubicin**
hsa-miR-135a-5p	TLE1	Predicted	Doxorubicin
hsa-miR-130a-3p	TLE1	Predicted	Doxorubicin
hsa-miR-9-5p	TES	Predicted	Doxorubicin
hsa-miR-17-5p	KIAA0922	Predicted	Carboplatin
hsa-miR-181b-5p	**EIF6**	**Predicted**	**Doxorubicin**
hsa-miR-451a	KIAA0922	Predicted	Carboplatin
hsa-miR-181b-5p	**TLE1**	**Predicted**	**Doxorubicin**

Table 4.10. Pathways enriched for COXEN genes predicted to interact with miRNAs associated with outcome in OS tumors (Based on Enrichr pathway analysis).

Pathway (WikiPathways2015)	P-value	Adj. P-val	Genes
Translation Factors (Mus musculus)	0.016	0.028	EIF6
Alpha6-Beta4 Integrin Signaling Pathway (Mus musculus)	0.022	0.028	EIF6
Translation Factors (Homo sapiens)	0.021	0.028	EIF6
Notch Signaling Pathway (Homo sapiens)	**0.021**	**0.028**	**TLE1**
Delta-Notch Signaling Pathway (Mus musculus)	0.028	0.028	TLE1

DISCUSSION

The goals of experiments described in this chapter were to utilize canine or human cell models to identify candidate miRNA biomarkers specifically associated with resistance to doxorubicin or carboplatin. We utilized a COXEN-approach to analyze both canine and publicly available human miRNA expression data and ultimately identify and select 20 miRNAs differentially expressed between doxorubicin and carboplatin sensitive or resistant cell lines. These miRNAs were subsequently evaluated using RT-qPCR in 33 canine osteosarcoma tumors

with recorded treatment and outcome data (COS33). In general, the pool of "drug-sensitivity-associated" miRNAs were not as highly associated with patient outcome as the pool of "tumor-outcome-based" miRNAs. Despite this challenge, when all miRNAs measured via RT-qPCR in the COS33 tumors were pooled and analyzed together, miR-98 emerged as contributing to predictive miRNA-expression-based signatures associated with patient outcome.

MiR-98 is a member of the let-7 family of miRNAs. The role of miR-98 in cancer appears to depend on the type of cancer and the genes targeted, as it has been reported as both a tumor suppressor and an oncogene.[12-14] Evidence of an association between elevated miR-98 and doxorubicin and cisplatin resistance has been established in squamous cell carcinoma and non-small cell lung cancer.[15, 16] Many of the effects of miR-98 in these human cancers were attributed to a direct interaction between mir-98 and high mobility group AT-hook 2 (HMGA2). Interestingly, in our tumor data we see elevation of miR-98 and loss of let-7b both associated with poor outcome. Further, both showed validated experimental evidence, according to multiMiR databases, for targeting testin LIM domain protein (TES), one of the genes dysregulated in the doxorubicin COXEN model from the Gustafson laboratory. TES is located on a common fragile site in humans associated with cancer and may inhibit cell growth or participate in cell adhesion (http://www.ncbi.nlm.nih.gov/gene). The interaction between let-7b, miR-98 and TES may suggest that changes in expression levels of one miRNA occurs in response to changes in TES protein levels caused by dysregulation of the other miRNA. Additional functional studies would be necessary to confirm these interactions, and any association with doxorubicin resistance in OS. Other work in our laboratory has confirmed overexpression of insulin-like growth factor two RNA binding protein 1 (IGF2BP1), a well established target of let-7b, contributes to doxorubicin resistance in canine and human OS cells.

We intend to validate the interaction of let-7b and IGF2BP1, further solidifying the potential link between low let-7b expression and doxorubicin resistance in OS.

Another objective for this chapter was to employ a COXEN-like analysis based on miRNA expression and utilizing RT-qPCR data for tumors. The advantages of such an approach should it be successful are 1) utilization of a platform with a lower price tag in hopes of making it available to a wider range of pet owners and 2) as a first step towards the ultimate use of miRNA expression in serum for COXEN analyses. Any identification of drug-associated biomarkers in the serum would be invaluable for monitoring therapy or early detection/analyses of metastases once the primary tumor is removed. However, there are several hurdles that would have to be overcome first.

One of the first limitations to our implementation of a COXEN model for doxorubicin was the restriction of all analyses to approximately 175 miRNAs successfully measured via RT-qPCR in a small set of tumors (n=14). The 190 original miRNAs were selected in part because of evidence, either in the literature or based on differential expression analyses in cell line data, of an association with drug sensitivity. However, successful implementation of COXEN using gene expression depended in part on starting with over 20,0000 genes in all datasets used throughout the process. Further, the gene expression datasets were all microarray based using arrays from the same manufacturer. Crossing platforms from arrays from different vendors to RT-qPCR data can be difficult due to different data processing and platform specific biases. In fact, it is encouraging that we started to see a trend toward separation of predicted sensitive and resistant tumors given all of the limitations we faced.

While our miRNA expression based COXEN model was not completely successful, we did find good candidate interactions between several of our dysregulated miRNAs and the genes

from the most predictive gene-expression-based COXEN doxorubicin and carboplatin models. One of those genes, transducing-like enhancer of split 1 (TLE1), is a transcriptional co-repressor that directly interacts with HES1 in the Notch signaling pathway during development.[17] TLE1 was over-expressed in our canine OS tumors compared to normal bone, and interestingly, over-expression of TLE1 occurs and is associated with resistance to doxorubicin in synovial cell sarcomas.[18] The potential role of down-regulated miRNAs that target TLE1, up-regulation of TLE1, dissociation of TLE1 and HES1 expression in aggressive OS tumors, and associations with doxorubicin resistance all warrant additional investigation.

Pulling together data from tumor-based and cell-based miRNA expression studies we identified five miRNAs whose dysregulation was most commonly associated with poor outcome in our 33 canine OS tumors: let-7b, miR-98, miR-130a, miR-181b, and miR-223. Pathway analysis with these 5 miRNAs identified enrichment of the PI3K-AKT pathway for genes targeted by all 5 of these miRNAs. Activation of the mammalian target of rapamycin (mTOR) pathway through PI3K-AKT occurs downstream of several tyrosine kinase receptors (RTKs) implicated in human and canine OS including platelet derived growth factor (PDGFR), erb-b2 receptor tyrosine kinase 2 (ERBB2) and insulin like growth factor (IGF1R).[19-23] Interestingly, the fifth most significantly enriched pathway identified in our pathway analysis was the insulin signaling pathway. Links between all of our top 5 miRNAs and the Notch signaling pathway and obesity or disruption of insulin metabolism can be found in the human literature.

MiR-98 and let-7b are both members of the Let-7 family and together with miR-181 can regulate and are regulated by lin-28 homolog (LIN28).[24-27] The link between the LIN28/Let-7 axis, and insulin metabolism is well-established and includes regulation of HMGA2, IGF2BP1 and IGF1R proteins and activation of PI3K-mTOR.[26] MiR-181 and miR-130 both potentially

target TLE1, the component of the Notch pathway implicated in COXEN models predictive of doxorubicin resistance. Notch also has a role in insulin metabolism[28], and interactions between Notch, PI3K-mTOR, and PTEN regulate cell survival and proliferation in T-cell acute lymphoblastic leukemia.[29] In addition to the let-7 family members, miR-130a and miR-223 both have established roles in regulation of obesity and insulin metabolism.[30] Our findings suggest that additional exploration of dysregulated insulin metabolism, including disruption of Notch and activation of PI3K-mTOR, in OS may reveal targets for novel treatment strategies.

Ours is not the only recent study incorporating genomic data and pathway analysis to also hone in on the PI3K/MTOR pathway in OS. A recent report by Perry et al combining sequencing of human and murine OS tumors with pathway analyses and a genome-wide functional shRNA screen in the mouse model of OS to identify genes "essential" for OS also converged on the PI3K/mTOR pathway as a key targetable pathway in OS.[31] Moriarity et al employed novel "Sleeping Beauty" forward genetic screens to identify over 200 common insertion sites (CIS) associated with OS development and 43 CIS specifically associated with metastasis.[32] Pathway analysis revealed CIS-associated genes were enriched in the ERRB, MAPK and PI3K/MTOR signaling pathways.[32]

Inhibitors of mTOR have been reported to have variable anti-tumor effectiveness *in vitro* and *in vivo* in both human and canine pre-clinical OS studies.[19, 33, 34] More recently, pre-clinical mTOR studies are incorporating newer, dual PI3K/MTOR inhibitors with promising results.[35] Three human trials incorporating mTOR directed treatment and including OS patients are listed on the www.clicaltrials.gov website. Results of the most recent of these described a phase II clinical trial evaluating ridaforolimus in patients with advanced bone or soft tissue sarcomas.[36] Two OS patients achieved confirmed partial responses, but results of the

study are difficult to interpret specifically for OS. Typical for human clinical trials, all patients were heavily pre-treated and likely entered this clinical trial only after their disease proved refractory to other standard of care treatments.

In summary, the data we present in Chapter 4 suggest that combining miRNA and gene expression data with predictive algorithms and pathway analyses in canine OS cells and tumors can be used to identify pathways associated with resistance to chemotherapy. In addition to identification of several targetable pathways on which to focus additional functional studies, we have identified miRNA biomarkers that might prove useful to stratify patients into groups most likely to benefit from novel treatment. Further, all of these pathways have also been implicated in human OS. Clinical trials using molecular biomarkers and predictive algorithms to direct chemotherapy and targeted treatment of canine OS will inform future clinical trials in human OS. Lastly, despite challenges associated with delivery of nucleic acid based therapies, rapid advances in therapies utilizing or targeting miRNAs may lead to incorporation of these agents in successful novel strategies for treatment of both canine and human OS.

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Chapter 5

General Conclusions

Osteosarcoma (OS), the most common primary bone tumor affecting canine and human patients, remains a clinical challenge. No appreciable improvement in cure rates has been achieved beyond advances associated with the integration of chemotherapy and surgical ablation more than 20 years ago. Clinical parameters do a poor job of predicting which of the estimated 80% of patients who present with undetectable metastases will ultimately fail conventional protocols. Human clinical trials to investigate incorporation of additional chemotherapeutics or novel therapies are hampered by slow accrual and challenges associated with determining effects of new drugs in heavily treated patients who have failed other therapies. The higher incidence of OS in canine patients, the similarity of the disease in both species, and the ability to potentially introduce novel therapies earlier in the course of treatment for canine patients makes canine OS an ideal translational model.

OS tumors are heterogeneous on many levels. They exhibit a complex karyotype and gene expression changes implicate disruption of numerous signaling pathways often with significant crosstalk between pathways. A single driver of OS progression remains elusive, and novel treatment strategies will more likely entail tailored treatment protocols incorporating combinations of targeted and conventional chemotherapies. Selection of novel therapies and the integration of these therapies into standard of care protocols requires identification of targetable pathways contributing to disease progression and metastasis in OS. Additionally, molecular biomarkers for prognosis, patient stratification, treatment selection and monitoring for relapse/metastasis will be important for the success of these efforts.

The studies presented in this dissertation represent our efforts to use and integrate gene and miRNA expression to identify disrupted pathways contributing to the progression of OS. We started in Chapter 2 taking a pathway specific approach using RT-qPCR and immunohistochemistry (IHC) to determine expression of key components of the Notch signaling pathway in OS tumors. Notch signaling is responsible for cell fate decisions during development and may contribute to maintenance of a stem cell like phenotype in cancer cells. Additionally, Notch is involved in regulation of the immune system and angiogenesis, processes that may contribute to metastasis. Notch inhibitors are currently in clinical trials and crosstalk between Notch and other targetable pathways including receptor tyrosine kinases is well documented. We demonstrated increased expression of four Notch pathway genes in tumors compared to normal bone, but found lower expression of hairy/enhancer of split-1 (HES1), a key Notch–activated transcriptional repressor, in tumors from patients with poor outcome. We confirmed this association between low HES1 expression and poor outcome using IHC in a larger set of independent tumors.

These findings led us to conclude that either Notch was not involved in progression of OS from local to metastatic disease or that some other pathway interrupted the connection between Notch and HES1 in the most aggressive tumors. In our efforts to explain the disconnect between Notch activation and low HES1 expression associated with poor prognosis, we found evidence for post-transcriptional HES1 regulation by miRNAs associated with the proliferative response of OS cells. Additionally, work in the laboratory focused on another dysregulated gene, insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) suggested escape from miRNA regulation as a contributing mechanism to the over-expression of this gene in aggressive OS. MiRNAs have proven attractive biomarkers due to their ability to simultaneously affect

multiple pathways and their increased stability in fixed tissues and circulating biofluids. Thus, we embarked on the mission of miRNA expression profiling in OS tumors and canine cancer cells to "flesh-out" the pathways contributing to OS metastasis and chemoresistance.

We first followed a similar pipeline to our gene expression studies, identifying candidate biomarker miRNAs by comparing expression of nearly 180 miRNAs in an updated DFI cohort of tumors and a set of normal bone samples with RT-qPCR. We then explored associations of 19 top candidates in a larger tumor set of 33 canine OS tumors. We identified several potentially predictive models, the best of which comprised a three-miRNA signature able to separate the tumors into high and low-risk patient subgroups with significantly different median survival times. MiRNA-mRNA interaction analyses of the most dysregulated miRNAs and genes in aggressive OS tumors suggested loss of miRNA regulation of IGF2BP1 expression as an important mechanism contributing to the aggressive OS phenotype.

Links between other miRNAs of interest and immune regulation, osteoclast differentiation and/or cytoskeletal adhesion protein regulation were also identified. Similar analyses between miRNAs and Notch-associated genes aberrantly expressed in tumors compared to normal bone identified numerous miRNA-mRNA interactions, which had been previously validated in other systems. The majority of these interactions were between miRNAs with lower expression and genes up regulated in tumors compared to normal bone, indicating general loss of miRNAs associated with regulation of Notch genes. Several implicated miRNA-mRNA pairs were part of targetable pathways affected by Notch including hedgehog, erb-b2 receptor kinase 2 (ERBB2) and poly (ADP-ribose) polymerase 1 (PARP1).

An underlying goal of miRNA biomarker discovery efforts in Chapter 3 was to identify miRNAs in OS patient serum associated with outcome. This strategy will become important for

monitoring patient status once the primary tumor is removed. Successful identification of serum-based biomarkers allow for less invasive sampling of a continuously renewable source. We were able to use a volume-based RT-qPCR strategy to identify a two-miRNA signature associated with patient outcome including decreased expression of miR-30c, which demonstrated similar expression changes in both serum and tumor samples.

Profiling miRNA expression in a panel of canine cancer cells allowed us to identify miRNA expression changes specifically associated with sensitivity to doxorubicin or carboplatin. While we were unable to replicate the success of the COXEN method using miRNA expression and RT-qPCR we were able to identify potential direct interactions between dysregulated miRNAs and genes from the doxorubicin and carboplatin COXEN models developed by the Gustafson laboratory. Further, this analysis brought our efforts to integrate different levels of genomic data and pathway analysis full circle, implicating dysregulation of the Notch pathway in doxorubicin resistance. Finally, taking data from Chapters 3 and 4 together, we chose 5 top miRNAs with expression changes associated with poor outcome in our OS tumors and found they all converged in the PI3K-AKT pathway. Inhibitors of this pathway are currently under investigation for treatment of refractory sarcomas, though progress is slow due to the previously described challenges associated with human clinical trials for rare tumors.

Future Directions

The work presented here has identified key gene and miRNA expression changes associated with outcome in OS and has implicated several signaling pathways associated with progression of disease and chemoresistance. Our work has confirmed the association between activation of Notch signaling and development of OS. By integrating miRNA and gene

expression data, we identified two additional avenues by which Notch may be influencing progression of OS. First, utilizing comparisons between tumors and normal bone, we identified several direct miRNA-mRNA interactions involving deregulation of Notch genes and associated with activation of other targetable pathways implicated in OS. Second, potential interactions between let-7b, miR-98, TLE1 and HES1 were identified which may contribute to doxorubicin resistance and metastasis. Comprehensive characterization of gene and miRNA expression in our canine OS cell lines will facilitate selection of appropriate cell models to further explore these interactions. These additional studies will validate interactions between miRNAs and target genes and determine the effect of manipulation of miRNA and target gene levels on cellular functions including proliferation, apoptosis, migration and invasion and sensitivity to chemotherapies.

We have identified seven miRNAs down regulated in aggressive OS tumors, which are predicted or known to target insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1). This knowledge will directly contribute to ongoing efforts in our laboratory to elaborate the role of IGF2BP1 in OS. This oncogene is known to have a long 3' UTR with alternative polyadenylation sites. Other work in our laboratory has demonstrated higher expression of IGF2BP1 with a shortened 3' UTR suggesting utilization of the more proximal polyadenylation site to escape miRNA regulation. Identification of miRNAs that can still bind this shorter 3' UTR may provide a potential mechanism for targeting this oncogene in the future.

Use of normal tissues, patient samples and cultured OS cell models in our analyses contributed to the identification of miRNA expression changes associated with poor outcome that likely depend on interactions with the tumor microenvironment. Expression of miR-223 is generally lower in OS cells and tumors than in normal bone. Yet, elevation of miR-223 along

with loss of miR-130a is associated with poor prognosis. Expression of these miRNAs is often associated with development of inflammatory cells, including differentiation of osteoclasts, which also arise from bone marrow monocyte precursors. Localization of miR-223 in OS tumors via fluorescent in-situ hybridization may provide some insight as to what types of cells are contributing to the elevation of miR-223 in OS tumors. Use of flow-cytometry in conjunction with subsequent quantitative analyses for measuring miR-223 in different cell populations would further enhance these efforts. The role of osteoclasts and macrophages in OS remains controversial, though several lines of evidence support activation of macrophages via compounds like muramyl tripeptide phosphatidylethanolamine (L-MTP-E) as a potential treatment for OS. These miRNAs may prove valuable biomarkers indicating tumors likely to benefit from this type of therapy.

The impetus for the studies included in this dissertation was to ultimately identify predictive biomarkers for use in improving prognosis and treatment planning in canine OS. Additional studies will be needed to validate the predictive capabilities of these biomarkers prospectively. Now that we have narrowed the focus to a handful of miRNAs of interest, TaqMan probes may be utilized to increase specificity of RT-qPCR for these efforts. Alternatively, direct quantitative methods such as generation of standard curves utilizing synthetic miRNAs, digital PCR or sequencing, which provide copy numbers of miRNAs in a given sample as opposed to relative expression, may provide additional validation for the more affordable platform we have utilized here.

The analyses here have expanded the picture of pathways associated with the progression of OS and more importantly, have generated a valuable data resource for more sophisticated bioinformatics analyses. For example, the described miRNA-mRNA interaction analyses here

elucidated only potential direct interactions between dysregulated miRNA and genes in OS. Yet we know regulation of genes via miRNA is much more intricate involving multiple levels of interactions and complex feedback mechanisms. Incorporation of network analyses of gene and miRNA expression data from canine OS cells and tumors will provide a more "three-dimensional" picture of biological networks driving OS.

Canine OS patients represent a valuable resource for overcoming some of challenges inherent in human clinical trials, particularly those involving rare cancers. Utilization of miRNA biomarkers, or incorporation of these biomarkers into predictive modeling strategies such as COXEN, will facilitate canine clinical trials in an individualized medicine setting. This will ultimately contribute to improved treatment strategies for both canine and human OS patients.

Appendix I

Table A.1 Primers used for miRNA RT-qPCR.

miRbase Name (v.21)	Primer Sequence
hsa-let-7a-5p	TGAGGTAGTAGGTTGTATAGTT
hsa-let-7b-5p	TGAGGTAGTAGGTTGTGTGGTT
hsa-let-7c-5p	TGAGGTAGTAGGTTGTATGGTT
hsa-let-7d-5p	AGAGGTAGTAGGTTGCATAGTT
hsa-let-7e-5p	TGAGGTAGGAGGTTGTATAGTT
hsa-let-7f-5p	TGAGGTAGTAGATTGTATAGTT
hsa-let-7g-5p	TGAGGTAGTAGTTTGTACAGTT
hsa-let-7i-5p	TGAGGTAGTAGTTTGTGCGTT
hsa-miR-1-3p	TGGAATGTAAAGAAGTATGTAT
hsa-miR-100-5p	AACCCGTAGATCCGAACTTGTG
hsa-miR-101-3p	TACAGTACTGTGATAACTGAA
hsa-miR-106a-5p	AAAAGTGCTTACAGTGCAGGTAG
hsa-miR-106b-5p	TAAAGTGCTGACAGTGCAGAT
hsa-miR-107	AGCAGCATTGTACAGGGCTATCA
hsa-miR-122-5p	TGGAGTGTGTGACAATGGTGTTTG
hsa-miR-125a-5p	TCCCTGAGACCCTTTAACCTGTGA
hsa-miR-125b-5p	TCCCTGAGACCCTAACTTGTGA
hsa-miR-128-3p	TCACAGTGAACCGGTCTCTTT
hsa-miR-128-2-5p	GGGGGCCGATACACTGTACGAGA
hsa-miR-129-5p	CTTTTTGCGGTCTGGGCTTGC
hsa-miR-130a-3p	CAGTGCAATGTTAAAAGGGCAT
hsa-miR-130b-3p	CAGTGCAATGATGAAAGGGCAT
hsa-miR-132-3p	CAGTGCAATGATGAAAGGGCAT
hsa-miR-133a-3p	TTTGGTCCCCTTCAACCAGCTG
hsa-miR-133b	TTTGGTCCCCTTCAACCAGCTA
hsa-miR-135a-5p	TATGGCTTTTTATTCCTATGTGA
hsa-miR-135b-5p	TATGGCTTTTCATTCCTATGTGA
hsa-miR-138-5p	AGCTGGTGTTGTGAATCAGGCCG
hsa-miR-141-3p	TAACACTGTCTGGTAAAGATGG
hsa-miR-142-3p	TGTAGTGTTTCCTACTTTATGGA
hsa-miR-142-5p	CATAAAGTAGAAAGCACTACT
hsa-miR-145-5p	GTCCAGTTTTCCCAGGAATCCCT
hsa-miR-146a-5p	TGAGAACTGAATTCCATGGGTT
hsa-miR-146b-5p	TGAGAACTGAATTCCATAGGCT
hsa-miR-148a-3p	TCAGTGCACTACAGAACTTTGT

hsa-miR-148b-3p	TCAGTGCATCACAGAACTTTGT
hsa-miR150-5p	TCTCCCAACCCTTGTACCAGTG
hsa-miR-152-3p	TCAGTGCATGACAGAACTTGG
hsa-miR-155-5p	TTAATGCTAATCGTGATAGGGGT
hsa-miR-15a-5p	TAGCAGCACATAATGGTTTGTG
hsa-miR-15b-5p	TAGCAGCACATCATGGTTTACA
hsa-miR-16-5p	TAGCAGCACGTAAATATTGGCG
hsa-miR-17-5p	CAAAGTGCTTACAGTGCAGGTAG
hsa-miR-181a-2-3p	ACCACTGACCGTTGACTGTACC
hsa-miR-181a-5p	AACATTCAACGCTGTCGGTGAGT
hsa-miR-181b-5p	AACATTCATTGCTGTCGGTGGGT
hsa-miR-181c-5p	AACATTCAACCTGTCGGTGAGT
hsa-miR-181d-5p	AACATTCATTGTTGTCGGTGGGT
hsa-miR-182-5p	TTTGGCAATGGTAGAACTCACACT
hsa-miR-185-5p	TGGAGAGAAAGGCAGTTCCTGA
hsa-miR 186-5p	CAAAGAATTCTCCTTTTGGGCT
hsa-miR-18a-5p	TAAGGTGCATCTAGTGCAGATAG
hsa-miR-18b-5p	TAAGGTGCATCTAGTGCAGTTAG
hsa-miR-18b-3p	TGCCCTAAATGCCCCTTCTGGC
hsa-miR-192-5p	CTGACCTATGAATTGACAGCC
hsa-miR-194-5p	TGTAACAGCAACTCCATGTGGA
hsa-miR-195-5p	TAGCAGCACAGAAATATTGGC
hsa-miR-196a-5p	TAGGTAGTTTCATGTTGTTGGG
hsa-miR-196b-5p	TAGGTAGTTTCCTGTTGTTGGG
hsa-miR-199a-5p	CCCAGTGTTCAGACTACCTGTTC
hsa-miR-199b-5p	CCCAGTGTTTAGACTATCTGTTC
hsa-miR-200a-3p	TAACACTGTCTGGTAACGATGT
hsa-miR-200b-3p	TAATACTGCCTGGTAATGATGA
hsa-miR-200c-3p	TAATACTGCCGGGTAATGATGGA
hsa-miR-202-3p	AGAGGTATAGGGCATGGGAA
hsa-miR-203a-3p	GTGAAATGTTTAGGACCACTAG
hsa-miR-205-5p	TCCTTCATTCCACCGGAGTCTG
hsa-miR-206	TGGAATGTAAGGAAGTGTGTGG
hsa-miR-208a-3p	ATAAGACGAGCAAAAAGCTTGT
hsa-miR-208b-3p	ATAAGACGAACAAAAGGTTTGT
hsa-miR-20a-5p	TAAAGTGCTTATAGTGCAGGTAG
hsa-miR-20b-5p	CAAAGTGCTCATAGTGCAGGTAG
hsa-miR-21-5p	TAGCTTATCAGACTGATGTTGA
hsa-miR-210-3p	CTGTGCGTGTGACAGCGGCTGA
hsa-miR-212-3p	TAACAGTCTCCAGTCACGGCC
hsa-miR-214-3p	ACAGCAGGCACAGACAGGCAGT

hsa-miR-217	TACTGCATCAGGAACTGATTGGA
hsa-miR-218-5p	TTGTGCTTGATCTAACCATGT
hsa-miR-22-3p	AAGCTGCCAGTTGAAGAACTGT
hsa-miR-221-3p	AGCTACATTGTCTGCTGGGTTTC
hsa-miR-222-3p	AGCTACATCTGGCTACTGGGT
hsa-miR-223-3p	TGTCAGTTTGTCAAATACCCCA
hsa-miR-224-5p	CAAGTCACTAGTGGTTCCGTT
hsa-miR-224-3p	AAAATGGTGCCCTAGTGACTACA
hsa-miR-23a-3p	ATCACATTGCCAGGGATTTCC
hsa-miR-23b-3p	ATCACATTGCCAGGGATTACC
hsa-miR-24-3p	TGGCTCAGTTCAGCAGGAACAG
hsa-miR-25-3p	CATTGCACTTGTCTCGGTCTGA
hsa-miR-26a-5p	TTCAAGTAATCCAGGATAGGCT
hsa-miR-26b-5p	TTCAAGTAATTCAGGATAGGT
hsa-miR-27a-3p	TTCACAGTGGCTAAGTTCCGC
hsa-miR-27b-3p	TTCACAGTGGCTAAGTTCTGC
hsa-miR-28-5p	AAGGAGCTCACAGTCTATTGAG
hsa-miR-296-5p	AGGGCCCCCCCTCAATCCTGT
hsa-miR-29a-3p	TAGCACCATCTGAAATCGGTTA
hsa-miR-29b-3p	TAGCACCATTTGAAATCAGTGTT
hsa-miR-29c-3p	TAGCACCATTTGAAATCGGTTA
hsa-miR-30a-5p	TGTAAACATCCTCGACTGGAAG
hsa-miR-30b-5p	TGTAAACATCCTACACTCAGCT
hsa-miR-30c-5p	TGTAAACATCCTACACTCTCAGC
hsa-miR-30d-5p	TGTAAACATCCCCGACTGGAAG
hsa-miR-30e-5p	TGTAAACATCCTTGACTGGAAG
hsa-miR-31-5p	AGGCAAGATGCTGGCATAGCT
hsa-miR-32-5p	TATTGCACATTACTAAGTTGCA
hsa-miR-320a	AAAAGCTGGGTTGAGAGGGCGA
hsa-miR-320b	AAAAGCTGGGTTGAGAGGGCAA
hsa-miR-320c	AAAAGCTGGGTTGAGAGGGT
hsa-miR-320d	AAAAGCTGGGTTGAGAGGA
hsa-miR-326	CCTCTGGGCCCTTCCTCCAG
hsa-miR-328-3p	CTGGCCCTCTCTGCCCTTCCGT
hsa-miR-331-3p	GCCCCTGGGCCTATCCTAGAA
hsa-miR-331-5p	CTAGGTATGGTCCCAGGGATCC
hsa-miR-335-5p	TCAAGAGCAATAACGAAAAATGT
hsa-miR-337-3p	CTCCTATATGATGCCTTTCTTC
hsa-miR-337-5p	GAACGGCTTCATACAGGAGTT
hsa-miR-339-5p	TCCCTGTCCTCCAGGAGCTCACG
hsa-miR-340-5p	TTATAAAGCAATGAGACTGATT

hsa-miR-346	TGTCTGCCCGCATGCCTGCCTCT
hsa-miR-34a-5p	TGGCAGTGTCTTAGCTGGTTGT
hsa-miR-34b-3p	CAATCACTAACTCCACTGCCAT
hsa-miR-34b-5p	TAGGCAGTGTCATTAGCTGATTG
hsa-miR-34c-5p	AGGCAGTGTAGTTAGCTGATTGC
hsa-miR-370-3p	GCCTGCTGGGGTGGAACCTGGT
hsa-miR-371a-5p	ACTCAAACTGTGGGGGCACT
hsa-miR-373-3p	GAAGTGCTTCGATTTTGGGGTGT
hsa-miR-374a-5p	TTATAATACAACCTGATAAGTG
hsa-miR-374b-5p	ATATAATACAACCTGCTAAGTG
hsa-miR-376a-3p	ATCATAGAGGAAAATCCACGT
hsa-miR-376b-3p	ATCATAGAGGAAAATCCATGTT
hsa-miR-376c-3p	AACATAGAGGAAATTCCACGT
hsa-miR-377-3p	ATCACACAAAGGCAACTTTTGT
hsa-miR-318-3p	TATACAAGGGCAAGCTCTCTGT
hsa-miR-421	ATCAACAGACATTAATTGGGCGC
hsa-miR-422a	ACTGGACTTAGGGTCAGAAGGC
hsa-miR-424-3p	CAAAACGTGAGGCGCTGCTAT
hsa-miR-424-5p	CAGCAGCAATTCATGTTTTGAA
hsa-miR-429	TAATACTGTCTGGTAAAACCGT
hsa-miR-431-5p	TGTCTTGCAGGCCGTCATGCA
hsa-miR-451a	AAACCGTTACCATTACTGAGTT
hsa-miR-454-3p	TAGTGCAATATTGCTTATAGGGT
hsa-miR-484	TCAGGCTCAGTCCCCTCCCGAT
hsa-miR-493-3p	TGAAGGTCTACTGTGTGCCAGG
hsa-miR-495-3p	AAACAAACATGGTGCACTTCTT
hsa-miR-497-5p	CAGCAGCACACTGTGGTTTGT
hsa-miR-503-5p	TAGCAGCGGGAACAGTTCTGCAG
hsa-miR-513a-3p	TAAATTTCACCTTTCTGAGAAGG
hsa-miR-513b-5p	TTCACAAGGAGGTGTCATTTAT
hsa-miR-513c-5p	TTCTCAAGGAGGTGTCGTTTAT
hsa-miR-519a-3p	AAAGTGCATCCTTTTAGAGTGT
hsa-miR-519b-3p	AAAGTGCATCCTTTTAGAGGTT
hsa-miR-519c-3p	AAAGTGCATCTTTTTAGAGGAT
hsa-miR-519-3p	CAAAGTGCCTCCCTTTAGAGTG
hsa-miR-519e-3p	AAGTGCCTCCTTTTAGAGTGTT
hsa-miR-520a-3p	AAAGTGCTTCCCTTTGGACTGT
hsa-miR-520b	AAAGTGCTTCCTTTTAGAGGG
hsa-miR-520c-3p	CTCTAGAGGGAAGCACTTTCTG
hsa-miR-520d-3p	AAAGTGCTTCTCTTTGGTGGGT
hsa-miR-520e	AAAGTGCTTCCTTTTTGAGGG

hsa-miR-520f-3p	AAGTGCTTCCTTTTAGAGGGTT
hsa-miR-520g-3p	ACAAAGTGCTTCCCTTTAGAGTGT
hsa-miR-520h	ACAAAGTGCTTCCCTTTAGAGT
hsa-miR-551a	GCGACCCACTCTTGGTTTCCA
hsa-miR-551b-3p	GCGACCCATACTTGGTTTCAG
hsa-miR-590-3p	TAATTTTATGTATAAGCTAGT
hsa-miR-630	AGTATTCTGTACCAGGGAAGGT
hsa-miR-654-3p	TATGTCTGCTGACCATCACCTT
hsa-miR-657	GGCAGGTTCTCACCCTCTCTAGG
hsa-miR-663a	AGGCGGGGCGCCGCGGGACCGC
hsa-miR-7-5p	TGGAAGACTAGTGATTTTGTTGT
hsa-miR-9-5p	TCTTTGGTTATCTAGCTGTATGA
hsa-miR-96-5p	TTTGGCACTAGCACATTTTTGCT
hsa-miR-96-3p	AATCATGTGCAGTGCCAATATG
hsa-miR-98-5p	TGAGGTAGTAAGTTGTATTGTT
hsa-miR-99b-5p	CACCCGTAGAACCGACCTTGCG
hsa-miR-99a-5p	AACCCGTAGATCCGATCTTGTG
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTGTG
hsa-miR-10b-5p	TACCCTGTAGAACCGAATTTGTG
hsa-miR-19a-3p	TGTGCAAATCTATGCAAAACTGA
hsa-miR-19b-3p	TGTGCAAATCCATGCAAAACTGA
hsa-miR-92a-3p	TATTGCACTTGTCCCGGCCTGT
hsa-miR-92b-3p	TATTGCACTCGTCCCGGCCTCC
hsa-miR-134-5p	TGTGACTGGTTGACCAGAGGGG
hsa-miR-154-5p	TAGGTTATCCGTGTTGCCTTCG
hsa-miR-301a-3p	CAGTGCAATAGTATTGTCAAAGC
hsa-miR-301b-3p	CAGTGCAATGATATTGTCAAAGC
hsa-miR-369-3p	AATAATACATGGTTGATCTTT
hsa-miR-382-5p	GAAGTTGTTCGTGGTGGATTCG
hsa-miR-544a	ATTCTGCATTTTTAGCAAGTTC