Breed-Associated MicroRNA Expression in Canine Osteosarcoma

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Abstract

MicroRNAs (miRNAs) are small non-protein coding RNAs that have been implicated in humans as having a fundamental role in cancer initiation and progression. Osteosarcoma
(OSA) is the most common bone tumor in dogs, however, little is known regarding mechanisms underlying malignant transformation in these tumors. Certain breeds such as Rottweilers and Greyhounds are at higher risk for developing OSA, suggesting that heritable factors play a role in this disease. We hypothesize that dysregulation of miRNAs in canine OSA is associated with specific breeds. In this study, we sought to characterize the expression of miRNAs in canine primary OSA tumors among Greyhounds, Rottweilers, Golden Retrievers, a mixed population of other breed and determine the association of differentially expressed miRNAs in primary tumors with breed. miRNA profiling of a panel of normal canine tissues revealed tissue-specific miRNA expression signatures. Independent real time PCR validation of a subset of tissue-specific miRNAs validated the use of the NanoString nCounter Assay as a platform for evaluating miRNA expression in canine tissues. Supervised hierarchical cluster analysis revealed distinct breed-associated miRNA expression signatures in canine OSA. 189 miRNAs were differentially expressed in Greyhound, Rottweiler, Golden Retriever, and Mixed Breed tumors (p<0.01). In an expanded cohort of Greyhound and Rottweiler tumors, real time PCR demonstrated that one of these, miR-494 (a miRNA with known importance in human tumorigenesis) is highly expressed in primary Rottweiler OSA as compared to Greyhound OSA (p<0.05) or normal canine osteoblasts cultured from various dog breeds, including one Rottweiler. These data reveal significant correlations between breed and miRNA expression in canine OSA, suggesting breed-associated patterns of miRNA dysregulation may play a role in the pathogenesis of this disease. Characterization of miRNA expression in canine osteosarcoma will facilitate
our understanding the biology of this disease and has the potential to identify
diagnostic/prognostic factors and targets for therapeutic intervention.

Introduction

Osteosarcoma is the most common primary bone tumor in dogs, representing
approximately 85% of all bone tumors with >10,000 new cases diagnosed each year.\(^{1,2}\) Both large and giant breed dogs are at a higher risk for the development of OSA, with
certain breeds such as Rottweilers and Greyhounds over-represented (10 fold increased
risk compared to other breeds) suggesting that heritable factors play a role in this
disease.\(^ {1,2}\) Canine OSA exhibits a similar clinical presentation to that found with
pediatric OSA including a primary tumor originating in the metaphyseal region of a long
bone and the presence of microscopic metastatic disease. Treatment of canine OSA
involves removal of the primary tumor, usually through limb amputation, although some
dogs undergo limb sparing surgery. Amputation alone results in a median survival time
of 3-4 months due to the rapid progression of microscopic metastases.\(^ {1,2}\) Survival times
are extended to 8-12 months if adjuvant chemotherapy with cisplatin, doxorubicin, or
carboplatin is used (reviewed in\(^ {2,3}\)). However, less than 20% of dogs will survive longer
than 2 years.

Historically, OSA has been the most intensively studied of the canine solid
tumors. Despite recent developments in understanding the molecular basis of
dysregulated expression of protein-encoding oncogenes and tumor suppressor genes such
as Met and PTEN, current therapy for OSA rarely leads to dogs surviving 2 years post-
diagnosis, with most patients succumbing to metastatic disease.\(^ {4,6}\) Until the molecular
alterations that underlie OSA are identified and characterized, developing more effective therapies will be challenging.

MicroRNAs (miRs) are a class of noncoding RNAs (19-24 nucleotides) that regulate gene expression at the post-transcriptional level through either mRNA cleavage and/or translational repression. MicroRNAs may regulate up to one-third of the human genes and thus play key roles in a wide variety of biological processes. They function by binding to conserved sequences within the 3’ UTR of mRNAs, thereby inducing the degradation of the target mRNA or inhibiting translation. Dysregulation of miRs is now a well-described phenomenon in human cancers where they function as either suppressors or promoters of tumor proliferation, differentiation and metastasis. Recently, miRNAs associated with human breast cancer were found to be similarly dysregulated in canine mammary tumors, suggesting that they may target conserved genes that initiate tumorigenesis or metastasis. These data suggest a fundamental role for miRNAs in cancer and highlight the potential of miRNA profiling as a diagnostic and prognostic tool and of manipulating miRNA expression for treatment.

We hypothesize that dysregulation of miRNAs in canine OSA is associated with specific breeds. In this study, we sought to characterize the expression of miRNAs in canine primary OSA tumors among Greyhounds, Rottweilers, Golden Retrievers, a mixed population of other breed and determine the association of differentially expressed miRNAs in primary tumors with breed.

The purpose of this study was to investigate the potential role of breed-associated miRNA dysregulation in canine OSA tumors among Greyhounds, Rottweilers, Golden Retrievers, and a mixed population of other breeds. In this study, we performed miRNA
expression profiling using the NanoString nCounter Assay, which we have validated as a platform for evaluating miRNA expression in canine tissues. Our data demonstrate that primary canine OSA exhibit unique miRNA expression signatures associated with specific breeds. MiR-494 expression is increased in primary Rottweiler OSA compared to primary Greyhound OSA and normal canine osteoblasts cultured from various dog breeds, including one Rottweiler. These data reveal significant correlations between breed and miRNA expression in canine OSA, suggesting breed-associated patterns of miRNA dysregulation may play a role in the pathogenesis of this disease.

Methods

Cell lines, primary cell cultures, primary tumor samples

Normal canine osteoblasts were generated from 5 different dogs and maintained in osteoblasts differentiating medium as previously described.\textsuperscript{12} Briefly, trabecular bone specimens from the femoral heads of canine patients undergoing femoral head osteotomy at the Veterinary Medical Center at The Ohio State University. The femoral head was washed repeatedly and a surgical curette was used to remove trabecular bone out of the femoral head. Bone chips were vigorously washed with sterile 1X Phosphate Buffered Saline (PBS). Following the washings, bone fragments were incubated in Dulbecco’s modified Eadle’s medium (DMEM)/F-12K medium (Specialty Media, Phillpsburg, NJ) supplemented with 50 μg/mL ascorbate, 256 U/mL collagenase type XI, 2 mM L-glutamine, and 50 μg/mL penicillin-streptomycin, and transferred to a spinner flask in a humidified incubator at 37°C with 5% CO\textsubscript{2} for 4 hours. Following digestion of cellular material, the bone chips were washed with 1X PBS, and plated into 75 cm\textsuperscript{2} polystyrene
cell-culture flasks (Corning Inc., Corning, NY) in osteogenic induction media consisting of DMEM/F-12K supplemented with 10% fetal bovine serum, 50 μg/mL ascorbate, 10 mM β-glycerophosphate, and 0.1 μM dexamethasone. Canine osteoblasts were evaluated for alkaline phosphatase (ALP) expression using a commercially available kit (Sigma) after 6 weeks in culture. Horse neutrophils served as a positive control and stem cells cultured in non-differentiating conditions served as a negative control. RT-PCR was performed on RNA from cultured osteoblasts to verify expression of characteristic markers of bone: Alkaline Phosphatase (ALP), Bone Morphogenic Protein-2 (BMP2), and Osteopontin (OP). Normal Osteoblasts (Cell App, Inc) were purchased and canine OSA8 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.

Normal tissues were obtained from 3 dogs and spontaneously occurring canine OSAs were obtained from 48 different affected dogs (12 Greyhounds, 12 Golden Retrievers, 12 Golden Retrievers, 12 mixed breed dogs) presented to the Veterinary Medical Center at The Ohio State University. Tissue samples were provided by the Ohio State University Veterinary Teaching Hospital Biospecimen Repository (OSU-VTHBR; Dr. Kisseberth, co-director). Collection for the OSU-VTHBR is IACUC approved 2009A0143. Fresh tissue samples were immediately flash frozen in liquid nitrogen and stored in The Ohio State University College of Veterinary Medicine Comparative Oncology Biospecimen Repository. All cases were previously diagnosed as canine OSA and clinical outcome data was available for all dogs.
NanoString profiling of mature miRNA expression in normal canine tissues and OSA biopsies

Total RNA was isolated by the Trizol (Invitrogen) method from normal canine liver, spleen, kidney, thyroid, brain cortex, lymph node and skeletal muscle from 3 healthy dogs and 48 primary canine OSA tumors from Greyhound, Golden Retriever, Rottweiler, and mixed breed dogs. RNA concentration was determined using the ND-1000 micro-spectrophotometer (NanoDrop Technologies).

Mature miRNA expression in canine tissues was performed using the NanoString nCounter human microRNA Expression Assay per the manufacturer’s instructions. This assay interrogates the expression profile of 752 human miRNAs; 168 of whose mature sequences are 100% conserved between human and dog (Sanger miRBase V15). Briefly, 100 ng total RNA per sample was hybridized overnight to the microRNA Expression Assay CodeSet and hybridized reactions were loaded onto the nCounter prep station for subsequent binding and washing steps. Cartridge scanning and quantification was performed with the nCounter Digital Analyzer. Nanominer software was used to perform raw data count normalization and p-values of <0.05 were considered statistically significant.

RNA isolation, reverse-transcription PCR and quantitative real time PCR

To determine miR-494 expression levels in available canine osteosarcoma and osteoblast cell lines, RNA was isolated using TRIzol (Invitrogen) from 8 canine osteosarcoma cell lines, a commercially available canine osteoblast cell line (CellApp, Inc), and primary canine osteoblasts obtained from 5 independent dogs (including
Greyhound, Rottweiler, Golden Retriever, and mixed breed dogs). Real-time PCR was performed using the Applied Biosystems StepOne Plus Detection System. Mature miRNA expression was performed using Taqman miRNA assays (Applied Biosystems). Briefly, 50 ng total RNA was converted to first-strand cDNA with miRNA-specific primers, followed by real-time PCR with TaqMan probes. Samples were normalized to U6 snRNA. All reactions were performed in triplicate wells and included no-template controls for each gene. Relative gene expression for all real time PCR data was calculated using the comparative threshold cycle method.\textsuperscript{13} Statistical analysis was performed using two-tailed paired Student’s $t$-test for qRT-PCR and p-values of $<0.05$ were considered statistically significant.

cDNA was synthesized from primary canine normal osteoblasts cultures, commercially available canine osteoblasts (Cell App, Inc.), and established canine osteosarcoma OSA8 cells using Superscript III (Invitrogen) per the manufacturer’s recommendations. Reverse-transcription PCR to detect the expression of bone markers ALP, OP, BMP2 was performed using ThermoScientific ThermoPrime Taq Polymerase.

**Statistical analysis**

Raw data count normalization and statistical analysis relative to miRNA expression data was performed with Nanominer software. The difference between normal canine tissues and comparisons among 4 breed groups were analyzed using multi-variate ANOVA analysis and p-values of $<0.05$ were considered statistically significant.

Real time PCR experiments were performed in triplicate and repeated 3 times. Data were presented as mean plus or minus standard deviation. The difference between 2
group means was analyzed using the Students $t$-test and a p-value of $<0.05$ was considered significant.

Results

**NanoString Technology human microRNA Assay Kit is a valid platform to evaluate microRNA expression in canine tissues**

The majority of identified miRNAs are highly evolutionarily conserved among many distantly related species, suggesting that commercially available human miRNA profiling assays may be used to evaluate miRNA expression in canine tissues. The NanoString human nCounter miRNA Assay is a hybridization-based quantitative assay that interrogates the expression profile of 752 human miRNA transcripts; 168 of whose mature sequences are 100% conserved between human and dog (Sanger miRBase V15). To determine if the NanoString nCounter assay can be used as a platform for evaluating miRNA expression from canine tissues, we performed miRNA profiling on a panel of normal canine tissues (liver, spleen, lymph node, brain cortex, thyroid, skeletal muscle, kidney) obtained from 3 healthy dogs. As shown in Figure 1, supervised hierarchial clustering of all 21 tissue samples revealed distinct tissue-specific miRNA expression signatures. We found 109 miRNAs were differentially expressed in normal canine tissues (Fig 1a).

Independent real time PCR was performed to validate the expression of a subset of tissue-specific miRNAs identified on the NanoString nCounter miRNA Assay. Real time PCR found high miR-1 expression in canine skeletal muscle, high miR-9 expression in canine brain cortex, high miR-122 expression in canine liver, and high miR-200c expression in
canine thyroid and kidney tissues (Fig 1b). These findings correlate with tissue-specific miRNA expression in human counterpart tissues and the mature sequences for these miRNAs are 100% conserved between human, dog, mouse, and rat. Independent real time PCR data from these canine tissue-specific miRNAs validates the NanoString human nCounter Assay Kit as a platform for evaluating miRNA expression in canine tissues.

**Breed-Associated MiRNA Expression in Canine Osteosarcoma**

To determine whether the influence of genetic background plays a role in canine OSA, we evaluated breed-associated miRNA expression in canine OSA from breeds with a higher incidence of OSA (Greyhound, Rottweiler, Golden Retriever) and a mixed population of other breeds. We evaluated miRNA expression using the NanoString human nCounter miRNA assay from forty-eight fresh frozen primary canine OSA biopsies from Greyhounds, Rottweilers, Golden Retriever, and mixed breed dogs. Supervised hierarchial clustering of all 48 tumors revealed distinct breed-associated miRNA expression signatures in canine OSA. We found 189 miRNAs were differentially expressed in Greyhound, Rottweiler, Golden Retriever, and Mixed Breed tumors (Fig 2).

**MiR-494 is Highly Expressed in Rottweiler Osteosarcoma**

In an expanded cohort of 20 Greyhound and 20 Rottweiler tumors, real time PCR demonstrated that miR-494 is highly expressed in primary Rottweiler OSA as compared to Greyhound OSA (Fig 5). To evaluate the potential role of miR-494 overexpression in canine OSA, primary normal canine osteoblasts were cultured from various dog breeds,
including breeds with a high incidence of OSA (1 Rottweiler, 1 Golden Retriever). Canine osteoblasts were generated in vitro from bone marrow derived stem cells collected from femoral heads and similar to established canine osteosarcoma (OSA8) and a commercial osteoblast (K9 Ob) cell line, these primary cultures expressed characteristic markers of bone (Alkaline phosphatase, Bone Morphogenic Protein-2, and Osteopontin) as detected by RT-PCR (Fig 4). Alkaline phosphatase expression was validated using immunocytochemistry staining (Fig 4). Real time PCR demonstrated that miR-494 was highly expressed in primary Rottweiler OSA as compared to normal canine osteoblasts, including osteoblasts from a Rottweiler dog.

**Discussion**

These data reveal significant correlations between breed and miRNA expression in canine OSA, suggesting breed-associated patterns of miRNA dysregulation may play a role in the pathogenesis of this disease. MiR-494, a miRNA with known importance in human oncogenesis, was found to be overexpressed in primary Rottweiler OSA as compared to primary Greyhound OSA and normal canine osteoblasts (including osteoblasts from a Rottweiler dog), suggesting that miR-494 overexpression may play a role in the pathogenesis of OSA in Rottweiler dogs. These findings highlight the role of heritable factors in this disease. Characterization of miRNA expression in canine OSA will facilitate our understanding the biology of this disease and has the potential to identify diagnostic/prognostic factors and targets for therapeutic intervention.

Future studies to evaluate the biological consequences of miR-494 overexpression on osteosarcoma and normal osteoblast cell behavior are currently underway. The canine
OSA16 and canine osteoblast cell lines, which express low levels of miR-494, will be stably transduced with pre-miR-494 lentiviral constructs. Functional assays evaluating the effect of miR-494 overexpression on cell proliferation, caspase 3,7 activity, matrix metalloproteinase expression, and matrigel invasion will be performed. In-parallel small RNA sequencing of canine primary OSA samples using the Applied Biosystems SOLiD 4 Sequencing platform is currently underway to validate the breed-associated miRNA expression signature in canine OSA.

References


Tissue-Specific Differential MicroRNA Expression in Normal Canine Tissues

Figure 1a. MiRNA expression profiling was performed on normal canine tissues obtained using the NanoString Technology nCounter human miRNA Expression Assay Kit. Hierarchical clustering revealed distinct, tissue-specific miRNA expression signatures. 109 miRNAs were differentially expressed and represent a tissue-specific miRNA expression signature (p<1e-06).

Figure 1b. Real time PCR was performed to independently validate the use of the nCounter System for evaluating miRNA expression in canine tissues.
Figure 2. MiRNA expression profiling on OSA tumors from Greyhounds, Golden Retrievers, Rottweilers, and a mix of other breeds revealed breed-associated miRNA expression; 44 miRNAs were differentially expressed among breed groups (p<0.001).
Figure 3. Hierarchical cluster analysis revealed 95 miRNAs differentially expressed among Greyhounds and Rottweilers (p<0.01).
K9 Ob  CBDC1 CBDC2 CBDC3 CBDC4 CBDC5 OSA8 (NTC)

ALP

BMP2

OP

GAPDH

*CBDC4 – Golden Retriever
*CBDC5 – Rottweiler

Alkaline Phosphatase

(+)

(-)

Canine Osteoblast  Canine Osteoblast
Figure 4. Canine osteoblasts were generated *in vitro* from bone marrow derived stem cells collected from femoral heads from 5 dogs. RT-PCR detected bone-specific transcripts in primary cultures, canine osteoblasts (K9Ob, Cell Applications, Inc) and canine OSA cells (OSA8). Alkaline phosphatase expression was assessed using a commercially available kit (Sigma). Upper left panel - horse neutrophils (positive control), upper right panel - stem cells cultured in non-differentiating conditions (negative control) and the lower two panels - ALP expression in differentiated canine osteoblasts.
5.1-fold higher expression in Rottweiler dogs vs. Greyhounds, p-value = 0.039

Figure 5. Real time PCR validation of miR-494 expression was performed on 20 Greyhound OSA, 20 Rottweiler OSA, and 5 normal canine osteoblast cell cultures. Rottweiler primary OSA demonstrated a 5.1-fold higher miR-494 expression as compared to Greyhound OSA (p-value=0.039) and was significantly higher than found in normal canine osteoblasts.