Title: DRIVERS OF MESENCHYMAL-EPITHELIAL TRANSITIONS IN SARCOMAS

Authors: Suzanne Bartholf DeWitt, DVM, Jason Somarelli, PhD, Mary Keara Boss, DVM, Sarah Wang, Alexander Hish, Shivee Gilja, Will Eward, DVM, MD

Background: Sarcomas are rare, yet very aggressive cancers of mesenchymal origin which can be highly migratory, invasive and metastatic. Several clinical studies have shown that ~~the overall survival of sarcoma~~ the transition to a more epithelial-like phenotype improves prognosis of sarcomas. We have identified a subset of canine sarcomas that express elevated levels of the epithelial biomarker, E-cadherin. Together, these observations led us to hypothesize that a mesenchymal-epithelial-like transition (MET) may reduce sarcoma aggressiveness. To test this hypothesis, we used combined expression of the microRNA200 family, and over-expression of the epithelial transcription factor GRHL2 to induce MET. MET induction significantly inhibited anchorage-independent growth and migration of sarcoma cells. We pinpointed the mesenchymal transcription factor and E-cadherin repressor, Zeb1, as a potential driver of the aggressive phenotype of sarcoma cells. Interestingly, over-expression of E-cadherin was also sufficient to reduce anchorage-independent growth of sarcoma cells, but did not induce MET. Together, our results suggest that MET may have prognostic significance by lowering anchorage-independent growth and migration of sarcomas. ~~patients is improved in patients with higher expression of epithelial biomarkers, such as E-cadherin, and worsened in patients with higher expression of mesenchymal biomarkers, such as Vimentin, and ZEB1. While mesenchymal-to-epithelial transitions (MET) of cellular phenotype appear to have some clinical relevance, the factors that mediate MET in sarcoma have not been identified. Preclinical data from our group suggests that MET can be induced in sarcoma cells with the combined over-expression of the epithelial-specific transcription factor, grainyhead-like 2 (GRHL2), and the miR200 family of microRNAs, inhibitors of the mesenchymal transcription factor, ZEB1.~~

Questions/Purposes: 1) ~~Does immunohistochemistry staining of E-cadherin and ZEB1 using naturally-occurring canine primary and metastatic sarcomas provide further evidence of this phenotypic plasticity~~Is phenotypic plasticity observed in clinical sarcoma specimens? 2) ~~Can expression of these genes be manipulated in vitro to induce MET in human sarcoma cells~~What are the factors and phenotypes that contribute to the improved prognosis of sarcomas that express a more epithelial-like biomarker profile?

Patients and Methods: Forty-six FFPE canine sarcoma samples, including 13 primary tumors with 13 matched metastases, were obtained from a clinical biorepository and stained by immunohistochemistry for E-cadherin and ZEB1. ~~**Scoring methods?** Additionally,~~ For preclinical experiments, human 143B osteosarcoma cells and RD rhabdomyosarcoma cells ~~in culture were transduced with a vector harboring the GRHL2 cDNA or with an empty vector control~~were stably transfected with E-cadherin and allowed to form colonies in soft agar. ~~We then used 2~~ In a parallel set of experiments, ~~independent siRNAs to knock down ZEB1~~ miR200s were reverse transfected with and without GRHL2 over-expression in these ~~2~~ two cell types ~~populations with or without GRHL2~~. ~~We extracted the RNA from these cells and performed qRT-PCR to compare expression levels of Zeb1 and E-cadherin.~~We compared the biomarker expression and cellular phenotypes (anchorage-independent growth and migration) of sarcoma cells with and without MET induction.

Results: We observed striking phenotypic plasticity in canine primary sarcoma tumors and metastases. ~~**(Better way to quantify this statement?)**~~ Interestingly, our preliminary analyses show that Zeb1 appears to be upregulated in metastases compared to matched primary tumors. In our *in vitro* studies, ~~ZEB1 knock down resulted~~ MET induction may inhibit sarcoma aggressiveness by reducing sarcoma cell anchorage-independent growth and migration.
~~in a significant increase in E-cadherin expression only in the context of GRHL2 over-expression.~~

Conclusions: Epithelial plasticity was observed in our canine sarcoma samples, suggesting that these transitions between states are clinically relevant. Our results suggest that ZEB1 and GRHL2 compete for occupancy of the E-cadherin promoter. Together, our data support the hypothesis that an epithelial-like

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state is prognostic for better outcomes in sarcoma patients. We have pinpointed E-cadherin and ZEB1 as drivers of these phenotypic transitions and mediators of sarcoma aggressiveness.

Figure 1. Phenotypic plasticity in canine sarcomas. A. E-cadherin expression is remarkably variable, with a subset of specimens demonstrating strong expression. **B.** The mesenchymal master regulator Zeb1 is upregulated in metastatic samples compared to matched primary tumors. ~~:~~

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~~**GRHL2 and ZEB1 compete for access to the E-cadherin promoter**~~