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REVIEW ARTICLE

A Contemporary Assessment of Osteosarcoma: Lessons from a Comparative Approach

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ABSTRACT

This review will describe more than two decades of comparative research on primary bone cancer (osteosarcoma). Osteosarcoma is a chaotic disease present in a complex and variable microenvironment composed of many different cell types which interact with each other and lead to high transcriptional heterogeneity. Despite this heterogeneity, common transcriptional patterns can be observed in the bulk transcriptomes of these tumors; additionally, these patterns are associated with outcome, indicating their importance to the molecular biology of the disease. Work from our group and others has led to our current understanding of osteosarcoma as a disease where multiple pathological processes appear to converge into a limited array of tissue organizations with distinct biology. Recurrent as well as distinct events can lead to these states of tissue organization, explaining the heterogeneity of osteosarcoma that is observed among and within species. Yet, despite their chaotic genomes, osteosarcomas seem to be (relatively) genetically stable, with persistent maintenance of essentially the same chromothriptic karyotype throughout the developmental lifetime of the tumor. Importantly, the transcriptional variance between tumors can highlight the underlying biology of the malignant cells themselves, as well as the composition of the osteosarcoma microenvironment and the host response, both of which are prognostically significant for this disease. Initial single cell RNA-seq reports provide further evidence of the importance of the osteosarcoma microenvironment for tumor characterization. Our data suggest that improving patient outcomes in immunologically barren or "cold" osteosarcomas, necessitates generating immune permissive or "warmer" microenvironments within the tumor. Furthermore, the aging bone microenvironment may create specific niches that predispose to cancer, and identification of the drivers that lead to these variable transcriptional patterns will be essential to identify personalized, effective genomic therapy for osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary tumor of bone, and it has been observed in skeletal remains across the tree of life[1,2]. Just as the maintenance of a hematopoietic progenitor population exposes weaknesses associated with impaired differentiation of blood cells leading to the occurrence of leukemias and lymphomas, maintenance of the mesenchymal stem cell population appears to present vulnerabilities leading to sarcomas including osteosarcoma. The difference is that bone precursors must be much more tightly regulated both spatially and temporally than blood precursors due to their roles in the formation of connective tissues.

Osteosarcoma is characterized by large chromosomal rearrangements. Even in more commonly occurring tumors, somatic driver events are obfuscated by the occurrence of large copy number changes, which can contain many individual genes in addition to the primary cancer driver(s). Using a comparative oncology approach, it has been proposed that the study of orthologous canine tumors can be used to isolate driver events to increase resolution due to syntenic differences between canine and human tumors. This approach is predicated on the idea that copy number change of regions containing specific driver genes is occurring under similar evolutionary conditions within the tumor despite the syntenic differences across species. However, we have recently recognized that different species have evolved different levels of cancer protective mechanisms[1], suggesting that tumor evolution may be occurring under different conditions in different species.

Despite significant efforts in the genomic era to understand osteosarcoma, minimal progress has been made in improving patient outcomes[3]. The following sections of this review will describe strengths and weaknesses of dog and mouse models osteosarcoma and how each has helped to improve our understanding of the molecular etiology this disease. It also raises a cautionary note by highlighting divergent features that suggest osteosarcomas in different species are convergent diseases that achieve common patterns of organization.

2. Comparative approach to the identification of the molecular etiology of osteosarcoma

2a. Animal models of osteosarcoma

By definition, osteosarcoma only occurs in vertebrate animals with ossified skeletons. This cancer is an ancient pathological entity: it has been found in a variety of animals that lived hundreds of millions of years ago, although it is almost certainly overrepresented in the fossil record because soft tissues decay more readily than bones. In the current era, osteosarcoma has been reported in individuals representing every vertebrate class. But overall, this tumor occurs only rarely across the whole of the vertebrate animal kingdom with the notable exception of domestic dogs where it is seen commonly, and especially among individuals from large and giant breeds[2].

As in most other vertebrates, osteosarcoma is a rare disease in humans with a peak incidence in adolescence. In dogs, the peak incidence is usually observed in adults comprising the oldest 25% of the population. Despite these different incidence patterns, the canine disease has been proposed as an ideal model to understand the human disease. The natural history of the canine disease is very similar to that seen in humans; the frequency with which osteosarcoma occurs in dogs provides ready access to samples for molecular and pathological studies and to canine patients for interventional studies; the tumors in dogs arise spontaneously (they are not induced), in an immunocompetent environment; and the treatment intensity and innovation applied to dogs with osteosarcoma are second only to humans[4].

As is true for many other tumors, mouse models have been foundational to our understanding of osteosarcoma biology[5-12]. These models are exceptionally tractable, but some do not recreate the anatomical distribution and metastatic patterns of human disease. One recent comparative approach that utilizes forward genetic screens in mice has been more successful in recreating the conditions observed in humans and has served to identify driver events associated with osteosarcoma[12]. In this model, osteosarcomas develop upon *Sleeping Beauty (SB)* mutagenesis of osteoblasts (**Figure 1**). These mouse tumors do not show the copy number changes observed in human and canine tumors; instead they are driven by mobilization of the *T2/Onc* transposon, allowing specific identification of driver genes. Genes identified by mutagenesis are often found in regions where somatic copy number changes are identified in naturally occurring tumors[12].

FIGURE 1

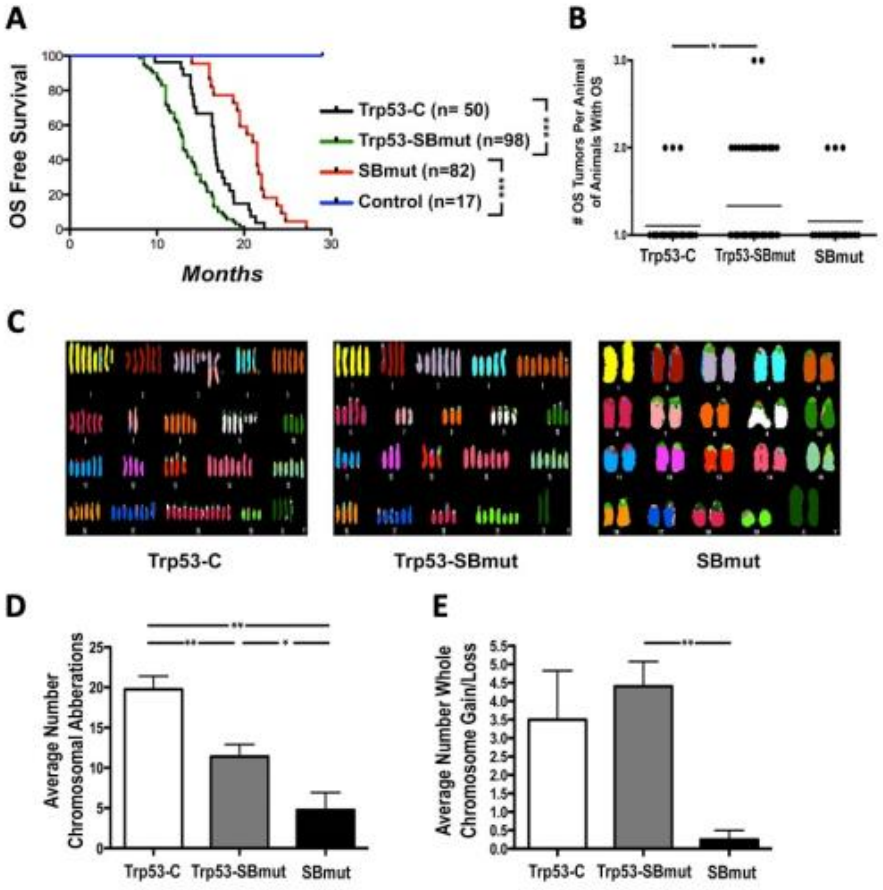

Figure 1. SB mutagenesis can accelerate or induce osteosarcoma development in cells with Sp7-cre expression. (A) Osteosarcoma-free survival curve depicting time to osteosarcoma development and survival endpoints in all cohorts. Control mice contained Sp7-cre with either SB11 or T2/Onc. ***P < 0.0001, log-rank test. (B) Histogram displaying the number of osteosarcomas per mouse. *P = 0.0159, Student's t test. (C) Representative SKY results from analysis of osteosarcoma tumor cells that developed in Trp53-C, Trp53-SBmut and SBmut mice. (D and E) Histograms demonstrating the number of chromosomal aberrations (E) and whole-chromosome gains and/or losses (E) identified by array CGH performed on Trp53-C (n = 4), Trp53-SBmut (n = 5) and SBmut (n = 4) osteosarcoma tumor DNA with matched normal tail DNA. *P < 0.05, **P < 0.001, Student's t test. Error bars, ± s.d. Adapted from ref[12].

Other animal models have been developed to study osteosarcoma, including zebrafish and pigs[13,14]. These models have unique strengths and open new avenues of investigation. But these alternative models have yet to achieve comparable abundance and maturity of data as mouse and canine models of osteosarcoma. And even as mice, dogs, and humans might share certain risk factors for osteosarcoma, there may be as many or more that are species specific (reviewed in[1,2,15]). A comparative approach has allowed us to recognize that the bone microenvironment is a highly complex tissue

and contains a variety of cells with complex cross talk and biological niches[16]. Species-specific differences have led us to conclude that there are multiple overlapping and distinct routes to osteosarcoma tumor formation and progression, and that osteosarcomas of humans, mice, dogs, and other animals are convergent diseases with complex etiologies that achieve comparable patterns of gross and histological organization.

It is thus clear that we must be diligent, deliberate, and vigilant to appreciate that animal models can be good or ideal in certain circumstances, while they may not be models at all in others.

2b. Disruption of *TP53*

Genomic efforts to understand cancer were initially focused on identifying somatic events that are recurrently observed in cancer tissue. In human osteosarcoma, the *TP53* gene has been observed to be recurrently disrupted using whole genome and exome sequencing of tumor tissue[17-20]. It seems apparent that loss of *TP53* function is associated with, and probably might be a major cause of the chaotic karyotypes seen in osteosarcomas[12]. Interestingly, it seems that once the chromothriptic event(s) have taken place, the tumor genomes can remain stable *in vivo*, even as they remain under strong selective pressure during progression and metastasis[21].

Highly recurrent disruption of *TP53* has also been reported in canine osteosarcoma[22-26]. Generally, higher rates of mutation have been reported in canine tumors than in human tumors, where structural variation is more commonly observed (**Figure 2**).

FIGURE 2

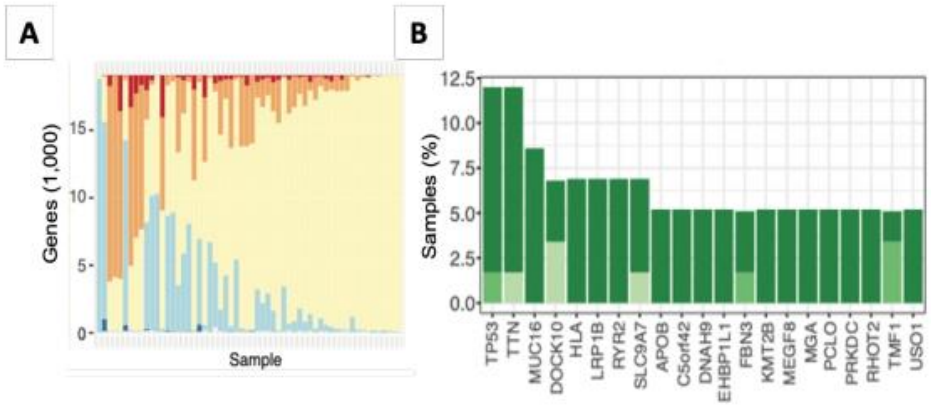

Figure 2. The mutational landscape of human osteosarcoma. We obtained tumor/normal exome sequence data for human osteosarcoma tumors (n=59) from GEO. (A) To examine copy number changes, we summarized the tumor normal reads obtained from the sequence capture to window segments and looked at the copy number changes within these windows. Expected copy number changes were observed on chrX and chrY. These serve as internal controls to show what loss of a single copy would look like (chrX) and also what complete loss of a region would look like (chrY). High level copy number gains were observed in MYC and RUNX2, and micro-deletions were present in regions containing CDKN2A, RB1, PTEN and TP53 corresponding to loss of either 1 or both copies of the chromosomal region. (B) Somatic mutations were identified utilizing a pipeline we designed to call mutations at specific locations based on the signal to noise ratios at each given position. Somatic mutations were observed in every tumor, although recurrence was low. Data are shown for the top-20 genes showing non-synonymous somatic mutations at any site as a proportion of all the samples analyzed. Many additional events were recurrently observed in a low percentage of tumors, but the majority were associated with very large genes that are mutated in many tumors without clear oncogenic functions.

Consistent with an important role for *TP53* in osteosarcoma, Li-Fraumeni syndrome predisposes carriers to osteosarcoma[27]. Germline variants in *TP53* have also been observed to be present in human osteosarcoma[28]. Recent work with human germline trios of affected progeny has shown that at least some of the germline mutations observed in human osteosarcoma cases are generated de novo, including mutations in *TP53*. That is, they are not present in the parents but are present in the germline of the affected patient[29]. While an analogous genetic disease to Li-Fraumeni has not been identified in dogs, a germline variant *TP53*

was reported in a dog that would be predicted to generate a frame shift variant[20].

2c. Loss of *CDKN2A*

The genomic region containing *CDKN2A* appears to be commonly lost in both human[20,30] and canine[31,32] osteosarcoma. Consistent with an important role for *CDKN2A* in osteosarcoma tumorigenesis, 2 independent case control studies identified markers near *CDKN2A* as the most significantly enriched region of the canine genome in dogs that developed osteosarcoma compared to dogs that did not[33,34]. Two distinct proteins with tumor suppressor function are generated from the *CDKN2A* locus, p16 and p14ARF. P16 regulates entry into G1 phase of the cell cycle through its interactions with CDK4/6 and *RB1*. P14ARF induces cell cycle arrest in G2 phase via binding the p53-stabilizing protein MDM2. Loss of *CDKN2A* has been shown to be an important event for malignant transformation of mesenchymal stem cells into osteosarcoma[35].

2d. Loss of *PTEN*

PTEN has been shown to be lost in human[20,36] and canine[32,37] osteosarcoma. Bi-allelic loss of *PTEN* occurs more commonly in the canine than in the human form of the disease (**Figure 3**)[i]. *PTEN* is a tumor suppressor that negatively regulates *PI3k* signaling. Meta-analyses of PTEN transcript levels show that increased levels of *PTEN* in tumors are associated with better outcomes[38].

[i] Sarver et al. Distinct mechanisms of PTEN inactivation in dogs and humans highlight convergent molecular events that drive cell division in the pathogenesis of osteosarcoma. Manuscript submitted.

FIGURE 3

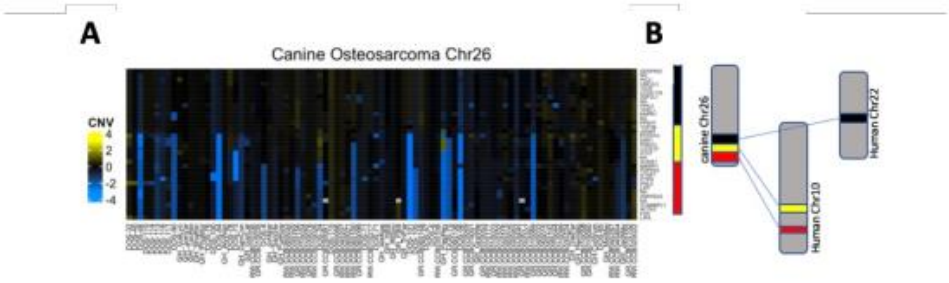

Figure 3. Loss of Distal Chr26 region containing PTEN in canine osteosarcoma. (**A**) Somatic changes observed between normal tissue and canine osteosarcoma tumors are shown for the distal end of canine chromosome 26. The data has been log_2 transformed and genes shown in bright blue represent somatic loss, genes shown in black are unchanged between tumor and control, and genes that show somatic gain are shown in yellow. (**B**) The syntenic alignment between canine chromosome 26 and the human genome is shown. The canine lost region is orthologous to two separate regions on human chr10 and one region on human chr22. Adapted from Sarver et al. Distinct mechanisms of PTEN inactivation in dogs and humans highlight convergent molecular events that drive cell division in the pathogenesis of osteosarcoma (manuscript submitted).

2e. Loss of *RB1, DLG2, and FAS*

RB1 is a tumor suppressor that negatively regulates the cell cycle via its interactions with multiple members of the network comprised by cyclin dependent kinase, (CDK), CDK inhibitors, and E2F transcription factors. *RB1* has been reported to be disrupted and lost in both human[20,39] and canine[32] osteosarcoma.

DLG2 is a tumor suppressor that has been reported to be mutated in human osteosarcoma[17] and to be lost in human and canine tumors[31].

FAS is a cell death receptor which is activated by *FASL*, leading to programmed cell death. Loss of *FAS* expression is one mechanism by which osteosarcoma cells may evade host resistance mechanisms in the lung, increasing metastatic potential[40-42]. In canine tumors loss of *FAS* occurs commonly due to homozygous deletion of the distal end of chr26, which also contains the *PTEN* gene[a].

Dogs that had lost Fas in their tumors showed improved therapeutic benefit from intratumorally delivered Fas ligand (FasL) under the control of a ubiquitin promoter and

encoded in an adenovirus vector[43]. The presumed mechanism of action was that FasL could interact with Fas on inflammatory cells without tumor cell apoptosis (as Fas-deficient cells do not undergo apoptosis in response to FasL). The subsequent death of these Fas-sensitive inflammatory cells established an environment that, somewhat paradoxically, promoted a self-amplifying cycle of inflammation and which led to innate and adaptive anti-tumor immunity[44].

2f. Gain of MYC

MYC is a prototypical oncogene involved in proliferation and growth. On its own in normal cells, *MYC* is tightly regulated, but in a permissive environment *MYC* is highly tumorigenic. Amplifications in the *MYC* region have been consistently reported in human[17,20] and in canine bone tumors[32,37].

3. Genetically engineered mouse models of osteosarcoma

Mice have been genetically engineered to generate osteosarcoma tumors. Germline mutation of *TP53* in mice leads to the generation of osteosarcoma tumors if they don't succumb to other tumors first. Targeting the *TP53* mutation to the osteoblast lineage drastically increases the frequency of osteosarcomas, and co-targeting *RB1* and *TP53* mutations within the osteoblast lineage accelerates tumor formation[9,10]. Targeting of *DLG2* in a *P53, RB1* background in the osteoblast lineage further accelerates tumor formation[31]. Conditionally targeting *PTEN* mutations with a *TP53* mutation in the osteoblast lineage also accelerates tumor formation[12]. But somewhat surprisingly, targeting *RB1* and *PTEN* together in the osteoblast lineage does not lead to osteosarcoma tumors, but instead leads to lipoma formation[45]. A number of other engineered mice develop osteosarcomas, including mice with *MYC* overexpression and *CDK2NA* loss (reviewed in[46]). These results consistently show that transformation of osteoblasts is a key step in the formation of osteosarcoma.

3a. Genetically engineered mice have been used to identify oncogenes and tumor suppressors in osteosarcoma

Forward genetic screens to accelerate tumor formation have been carried out in osteoblast lineage cells using the *Sleeping Beauty* transposon to mobilize a *T2/ONC* transposon (**Figure 1**)[12,47]. These screens have identified important roles for *RB1*, *MYC*, *PTEN*, and *CDKN2A* in osteosarcoma, genes which have been shown to be modified by copy number change or mutation in human and canine tumors.

4. Comparative analysis identifies disruption of the *PI3K* signaling pathway in osteosarcoma

Enrichment analyses of the universe of somatic events observed in human patients point to disruptions of the *PI3K/mTOR* pathways in osteosarcoma[19]. An siRNA screen for essential genes in a mouse osteosarcoma cell line also identified key roles for cell cycle genes, as well as *PI3K/mTOR* pathway genes[19]. Analyses of the combined set of genes identified by forward genetic screens

for osteosarcoma showed pathway enrichment for the *PI3K* signaling pathway[12] as well as cell cycle genes. These independent observations support the importance of the *PI3K* signaling pathway in osteosarcoma tumorigenesis.

5. Syntenic synergy in osteosarcoma

Colocalization of genes within oncogenic risk neighborhoods may play an important role in osteosarcoma and other tumors when they are commonly lost. *PTEN* and *FAS* are commonly lost in canine osteosarcomas, leading to a tumor with diminished suppression of PI3K signaling and decreased apoptotic capability in the presence of *FASL*. The genomic susceptibility to biallelic loss of these two genes, found on the distal end of chr26 within the canine genome, may partially explain the increased risk as well as the shorter survival times associated with canine osteosarcoma[ii].

Other regions of the genome may also carry syntenic synergistic risk and vulnerabilities. For example, *MTAP* is located near *CDKN2A* and is commonly lost leading to tumorigenic changes in metabolism that may provide competitive advantages to tumor clones beyond loss of *CDKN2A* derived protein products. Cells that have lost *MTAP* require *PRMT5*[48] and are sensitive to treatment using *PRMT5* inhibitors[49]. *MYC* and *PVT1* are also commonly amplified together leading to synergistic interactions and increased transformation relative to the individual components[50]. Synergistic interactions based on gene synteny may be important to understanding species specific cancer risk as well as defining weaknesses specific to cancer cells.

6. Comparative genomic studies of osteosarcoma transcriptional patterns

In contrast to other tumors like colon, breast, or blood tumors, inter-tumor transcriptional heterogeneity is extremely high in osteosarcoma tumors. Recent work using single cell analyses of osteosarcoma tumor cells suggests that the expression differences observed between osteosarcoma tumors are likely due to the presence of different populations of tumor and stromal cells at different differentiation states within the bulk microenvironment[51]. Recent work utilizing spatial transcriptomics suggest that multiple distinct cytokine mediated microenvironment niches exist within the bone and bone marrow which allow for the mesenchymal stem cell lineage cells to interact with and regulate the hematopoietic stem cell system[52]. Osteosarcoma may arise from deregulation of these niches and cellular imbalances may be generating the high level of variance observed with bulk osteosarcoma sequencing. This, coupled with difficulties in extracting RNA from bone tissues, has slowed advances in understanding transcriptional heterogeneity in osteosarcoma. Despite these

[ii]Sarver et al. Distinct mechanisms of PTEN inactivation in dogs and humans highlight convergent molecular events that drive cell division in the pathogenesis of osteosarcoma. Manuscript submitted.

complications, a number of landmark studies have identified conserved transcriptional patterns in mRNA and miRNA to be present across sets of osteosarcoma tumors derived from humans, mice and canines and these conserved patterns have been associated with patient outcome.

The gene cluster expression summary score (GCESS) has been used to systematically independently identify and quantify sets of coregulated genes in human, canine and murine tumors[53]. The GCESS is defined as the sum of expression values (log_2-transformed and mean centered) of all genes in a particular defined cluster for a single sample. Clusters of genes are defined by a minimum average linkage hierarchical clustering threshold and a minimum gene number in order to identify strong patterns in the data. Essentially, the GCESS method carries out dimensional reduction of many correlated individual transcript data points and condenses them into a single value. The GCESS method can then be used for statistical analyses of the gene expression patterns with reduced noise relative to individual transcripts. Cluster membership can then be compared across datasets and across species identifying orthologous transcriptional patterns (**Figure 4**).

FIGURE 4

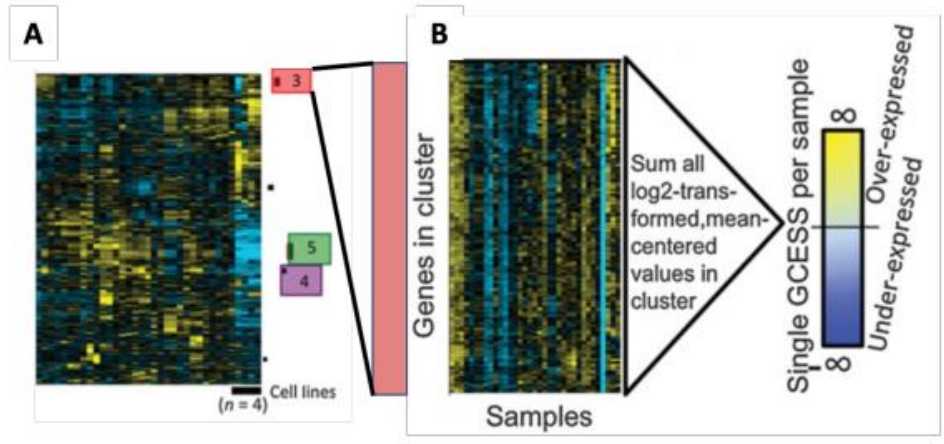

Figure 4. Schematic illustration of the GCESS method. The GCESS method was developed to reduce the high dimensionality of expression profiles generating a normalized and easily comparable value per sample for use in further association analyses. The GCESS is defined as the sum of expression values (log 2 -transformed and mean centered) of all genes in a particular gene cluster for a single sample. A negative GCESS indicates relative under-expression of the group of genes in that sample compared with all of the samples in the analysis set, a positive GCESS indicates overexpression, and a GCESS close to 0 (zero) indicates mean expression. The

GCESS summarizes the relative transcript levels of many correlated genes into a single value. This value is calculated for each cluster in each tumor. This allows for tumors to be rank ordered by summary score, which is based on the observed transcriptional data. Multiple summary scores were generated for each tumor sample, allowing for the independent comparison of the impact of each identified gene cluster, thereby achieving an unbiased dimensional reduction. Adapted from ref[53].

Using the GCESS approach, conserved transcriptional variation has been observed in human, mouse, and canine osteosarcomas in a cluster of genes associated with cell cycle progression[53,54], a cluster of genes associated with immune cell infiltration[53] and a set of miRNA present at the 14q32 human locus[55]. Increased transcription associated with cell cycling has consistently been associated with poor outcome in human and canine osteosarcoma[53,54,56] as well as other tumor types[53,56]. Variable expression of components of the immune system were identified in human mouse and canine samples[53]. Decreased expression of transcripts associated with immune infiltration has been associated with poor patient outcomes[53]. Decreased expression of a set of co-clustered miRNA at the 14q32 locus was observed in human, canine, and mouse samples[57] and was also associated with poor outcome in human and mouse samples[58]. These results have been validated in additional sets of human tumors[59].

7. Linkage of driver events to transcriptional patterns

Identification of driver events responsible for transcriptional heterogeneity of tumors has been difficult in osteosarcoma for a number of reasons. First, driver events outside of *TP53* are highly heterogenous in osteosarcoma. Second, osteosarcoma RNA-seq datasets are generally relatively small compared to other tumors studied. Third, RNA-seq dataset quality is also influenced by difficulties in the extraction of mRNA from bone samples. Fourth, each tumor likely has a distinct background of additional somatic events and germline variants that are likely to modify the transcriptional response of a given driver event. Despite these difficulties, using comparative approaches, driver events can be linked to the observed transcriptional patterns using GCESS based analyses.

As noted above, much higher rates of bi-allelic *PTEN* loss have been reported in canine than in human osteosarcoma. This has allowed us to elucidate the role of *PTEN* in tumor transcriptional heterogeneity. In canine tumors, loss of *PTEN* DNA can be directly correlated to loss of *PTEN* transcript. *PTEN* transcript levels are highly negatively correlated with the cell cycle GCESS in four independent RNA-seq datasets. Taken together these results strongly suggest that *PTEN* loss is a cause of increased cell division in canine osteosarcoma. In human osteosarcoma, increased methylation of the 5' shoulder region of the CPG island in the *PTEN* promoter is associated with increased cell cycle scores. Rather than directly controlling

the basal transcript level of *PTEN*, methylation in human and DNA loss in canines likely inhibits the inducibility of *PTEN* in response to inappropriate cell division. Distinct mechanisms of *PTEN* inactivation in dogs and humans highlight convergent molecular events that drive cell division in the pathogenesis of osteosarcoma[iii].

Mechanisms by which immune infiltration is controlled in osteosarcoma also remain murky. Microenvironment niches have been described within the bone marrow environment for the regulated proliferation and differentiation of hematopoietic stem cells[52,60]. Loss of immune infiltration within the tumor microenvironment suggests that mechanisms exist for maintaining immune cell presence in normal bone, which are compromised in osteosarcoma. Evidence for crosstalk between tumor cells and the immune system also exists in retinoblastoma, where a normally immune privileged environment generates an immune response following inappropriate proliferation. This immune response is negatively correlated to cell cycling in human tumors as well as genetically engineered mouse tumors[61].

RNA-seq analyses of SB mutagenized screens provide clues to how the immune system is deregulated in osteosarcoma. Tumors that overexpress *CSF1R* have very low levels of immune infiltrate. The addition of *CSF1R* overexpression in *TP53* mutant osteoblasts accelerates tumorigenesis in genetically engineered mice and the resulting tumors show lower levels of immune infiltrate relative to *TP53* mutation alone. It is likely that *CSF1R* is hijacking the *CSF1* cytokine creating niches that do not support proliferation of the immune system[iv].

Evidence also exists that an increased immune response due to infection is associated with better outcomes in osteosarcoma. Dogs with post-operative wound infections after limb-salvage surgery for osteosarcoma have improved survival[62]. In human patients, deep post-operative infections also lead to better outcomes[63]. These results suggest that mechanisms that recruit additional immune cells may be beneficial to osteosarcoma.

8. Immunotherapy for osteosarcoma

Dogs as a model have their own limitations[2]; however, we and others have identified instances where the biology allows us to utilize dogs to advance immunotherapy for this disease (reviewed in ref.[64]). The canine trials conducted to date have had mixed results, but a consistent conclusion from these studies is that immunotherapies that activate innate immunity improve survival for dogs with non-metastatic appendicular osteosarcoma[43,65,66]; this has also been

[iii] Sarver et al. Distinct mechanisms of PTEN inactivation in dogs and humans highlight convergent molecular events that drive cell division in the pathogenesis of osteosarcoma. Manuscript submitted.

[iv] Sarver et al. CSF1R regulates immune infiltration in the pathogenesis of osteosarcoma. Manuscript submitted.

observed in humans[67]. Less work has been done in the metastatic setting, where the benefits remain unclear[68]. A recently published study showed benefit of inhaled IL-15 in dogs with advanced metastatic osteosarcoma[69], and a new study combining an oncolytic vesicular stomatitis virus (VSV)[70] and a novel peptide designed to simultaneously block the CD47/SIRPα myeloid checkpoint and the PD-1/PD-L1 immune exhaustion checkpoint, is underway at our institution (z.umn.edu/METEOR). Conventional strategies using antibodies to achieve blockade of the CTLA4 and/or the PD-1/PD-L1 immune exhaustion checkpoints have yet to be completed in canine osteosarcoma. But studies incorporating PD-1/PD-L1 checkpoint blockade have shown no (or very limited) success in human osteosarcoma[71,72].

9. Conclusions

Osteosarcoma is a chaotic disease present in a complex and variable microenvironment composed of many different cell types which can interact with each other leading to high transcriptional heterogeneity. Despite this heterogeneity, common transcriptional patterns can be observed in the bulk transcriptomes of these tumors; additionally, these patterns are associated with outcome, indicating their importance to the molecular biology of the disease. Deconvolution of the cell types present in osteosarcoma will be important to identifying compounds that modify bone marrow niches. While there are clear similarities between highly conserved driver events (*e.g.*, *TP53* mutation across species) conservation of molecular etiology is not absolute across species as is exemplified by PTEN (silenced via methylation in humans vs biallelic loss in dogs). Despite these potential pitfalls a comparative oncology approach has high value to the study of osteosarcoma. Naturally occurring canine tumors and genetically engineered mouse models allow opportunities to study osteosarcoma in the presence of a functional immune system which are not possible with the commonly used osteosarcoma cell line models. We suspect that targeting specific therapies to specific combinations of transcriptional patterns may improve outcomes. For example, the comparative study of human and canine tumors suggests that to improve patient outcomes in immune "cold" osteosarcomas we should be focusing therapeutic strategies on approaches that generate immune "warmer" microenvironments. We suspect that the aging bone microenvironment may create specific niches that predispose to cancer, perhaps through loss of immune monitoring, changes to the immune system as a result of aging, niche modification leading to changes in cellular composition, or by combination of these events. Identification of the molecular drivers that lead to these variable tumor specific transcriptional patterns will be essential to develop more personalized genomic therapy for osteosarcoma. Identification and remedy of factors that lead to immune deficiency in individual tumors may also lead to principles that guide general one-size-fits-all pharmaceutical intervention to activate and improve tumor immune response in osteosarcoma.

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