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Osteosarcoma Genetics and Epigenetics: Emerging Biology and Candidate Therapies

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Abstract

Osteosarcoma is the most common primary malignancy of bone, typically presenting in the first or second decade of life. Unfortunately, clinical outcomes for osteosarcoma patients have not substantially improved in over 30 years. This stagnation in therapeutic advances is perhaps explained by the genetic, epigenetic, and biological complexities of this rare tumor. In this review we provide a general background on the biology of osteosarcoma and the clinical status quo. We go on to enumerate the genetic and epigenetic defects identified in osteosarcoma. Finally, we discuss ongoing large-scale studies in the field and potential new therapies that are currently under investigation.

Keywords

epigenetics; genetics; metastasis; osteosarcoma; therapy

I. INTRODUCTION

Osteosarcoma is a rare mesenchymal tumor that is histologically characterized by the presence of malignant mesenchymal cells and the production of a bone stroma. Within this histological diagnosis, distinct histological subtypes have been defined, including osteoblastic, chondroblastic, fibroblastic, and telangiectatic osteosarcomas.¹ Nonetheless, the biological behavior of and treatments for these distinct subtypes are similar. In the United States there are approximately 900 new cases of osteosarcoma reported each year.² Despite its rarity, however, osteosarcoma represents the most common primary malignancy of bone. The biology of this tumor is most commonly characterized by an appendicular primary tumor with a high rate of metastasis to the lungs.³ This review summarizes the biological behavior of this tumor and conventional approaches to therapy. Given the stagnation in clinical outcomes seen with such conventional therapies over the past 3 decades, a focus of this review is the ongoing efforts to define the genetic and epigenetic changes that are associated with tumor formation and those associated with progression and

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metastasis. Using these data, we propose a biological rationale to consider novel and targeted therapies for osteosarcoma.

II. PATHOGENESIS, BIOLOGY, AND CONVENTIONAL THERAPY

Genetic aberrations or changes in gene expression in osteosarcoma tissues have not identified common or recurrent genetic lesions or pathway alterations that explain the development of this tumor type.^{4,5} Rather, osteosarcoma is best characterized by its disorganized genome. Indeed, the most consistent genetic finding in osteosarcoma, beyond dysregulation of p53 and Rb (retinoblastoma), is significant aneuploidy and some evidence of massive disruption in the control of chromosomal structure (i.e., chromothripsis).^{5,6} This has suggested the possibility of an early defect in DNA repair/surveillance as a mechanism for osteosarcomagenesis and the resultant bizarre aneuploidy.

A. Cell of Origin

Beyond the difficulty in identifying recurrent and driving genetic alterations associated with osteosarcoma development, the cell of origin for this tumor type remains unclear.⁷ Historically, cells committed to the bone lineage (e.g., the osteoblast and preosteoblast) were reasonably believed to represent a cellular target for transformation.⁸⁻¹⁰ More recent data have been used to suggest the hypothesis that the osteosarcoma-transforming event occurs in multipotent mesenchymal stem cells, and that this event then yields a karyotypic complexity that is tolerated by these primitive mesenchymal cells, subsequently driving them down bone-differentiation lineages.^{11,12} Irrespective of the actual cell of origin, it is commonly agreed that the gene expression and the cellular phenotype of osteosarcoma are related to bone.¹³⁻¹⁵

Accordingly, the characteristic presentation of osteosarcoma indicates a tight association with normal phases of skeletal development. Osteosarcoma most often develops in the appendicular skeleton in pediatric patients, during the second decade of life. The timing of tumor diagnosis in patients coincides with the developmental growth spurt that occurs in this patient population.¹⁶ This epidemiological association as well as both cellular and molecular data have suggested a role for skeletal growth regulating the growth hormone–insulin-like growth factor axis in the development and progression of osteosarcoma.¹⁷

B. Metastasis and Progression

The metastatic cascade represents a series of processes that occur as a cell leaves the primary tumor and invades the surrounding tumor microenvironment, leading to intravasation into existing or new vascular structures, survival in the circulation, and eventual arrest and extravasation at distant secondary sites, followed by the development or recruitment of a blood supply and growth at the secondary site.¹⁸ Successful metastatic cells must detect, modulate, and manage discrete cellular stresses to metastasize successfully.¹⁹⁻²² A critical and potentially defining determinant of metastatic success that has been observed in many cancers is their ability to overcome the substantial stress experienced as cells engage the microenvironment of the secondary site.^{19,20,23} Cells must rapidly adapt to this new and hostile environment to survive. This is important as tumor cells

first enter a secondary metastatic environment but also as cells reengage the microenvironment later during metastatic progression. We and others believe the stresses faced by metastatic cells are distinct from those experienced during initial tumor formation. Our understanding of the timing of events leading to metastatic progression, and the events themselves, are far from complete. For example, it is unclear whether microscopic cells that leave the primary tumor early in the development of osteosarcoma move directly to the lung and reside there for long periods of time (dormancy), or whether these cells transit from the primary tumor to “protected environments,” where they become dormant and then move through all the steps of metastasis en route to the lung.²⁴ Similarly, it is reasonable that the cells capable of completing the complex set of events required for metastasis continue to metastasize to more distant sites (within the lung in osteosarcoma).²⁵ Indeed, if metastatic cells emerge from “protected sites,” and if metastatic lesions continue to metastasize, the opportunity to improve patient outcomes by targeting the process of metastasis would have merit not only in patients who present with localized and presumed microscopic metastasis but also in patients presenting with gross metastasis. Shortcomings in our understanding of the biology of metastasis preclude any a priori judgments of the value of agents that target any aspect of the process of metastasis as potentially valuable in osteosarcoma. Rigorous preclinical studies and innovative clinical trial designs, however, are necessary to allow the assessment of agents that target metastatic progression in patients. This is particularly true in the case of osteosarcoma because outcomes for these patients have not improved for more than 3 decades.³ Many of the aberrant genetic and epigenetic mechanisms leading to the development of osteosarcoma tumors are reasonably expected to contribute to metastatic progression. These mechanisms are summarized in figure 1, which is meant to serve as a visual reference for the descriptions in the text to follow.

C. Conventional Treatment and Outcomes

Approximately 80% of patients present with grossly localized disease.²⁶ Accordingly, definitive resection of the primary tumor is necessary, and this is readily achieved in most patients.³ Neoadjuvant chemotherapy has been incorporated into many treatment protocols. The use of neoadjuvant chemotherapy has many goals, but, most clearly, it seems to simplify surgical resection.²⁷ Assessment of tumor necrosis following neoadjuvant therapy can be prognostically valuable,²⁸ and it can also be used to prioritize chemotherapeutics that may be best used in the adjuvant setting. Nonetheless, it is unclear whether the use of neoadjuvant therapy improves long-term outcomes for patients beyond improving the management of the primary tumor. Despite the localized presentation for most patients, the successful resection of most primary tumors, and both neoadjuvant and adjuvant chemotherapy, many patients (35%) develop metastases.^{28,29} This is certainly the result of microscopic metastases that were not detectable at the time of presentation. Among patients who present with disseminated disease, over 80% progress within 5 years.³ Long-term outcomes for patients who present with localized or disseminated disease have largely remained unchanged over the past 30 years.³ Greater intensification of cytotoxic therapy alone is not likely to improve these long-term outcomes. Accordingly, progress is needed to define recurrent genetic or epigenetic alterations linked to osteosarcomagenesis and, perhaps more important, linked to osteosarcoma progression and metastasis.

III. GENETICS

A. Genetic Susceptibility

1. Genome-wide Association Studies—The past decade has seen an explosion of studies seeking to understand how genetic variability contributes to disease. These population-based studies, known as genome-wide association studies, seek to detect genetic variants—most commonly single nucleotide polymorphisms (SNPs)—that are associated with complex traits in populations (e.g., susceptibility to cancer).³⁰ Recent studies of humans and dogs with osteosarcoma revealed multiple SNPs associated with risk for the development of osteosarcoma.^{31,32}

Numerous studies associating common genetic variants with osteosarcoma risk have been published in the past 15 years.^{33–39} While in these studies risk SNPs have been linked to biological pathways with known relevance to osteosarcomagenesis, their statistical power has been limited by a small sample size because of the rarity of this cancer type. A recently published study sought to overcome such limitations through an international collaborative effort comparing genotypes of 941 human osteosarcoma cases with those of 3291 controls. Data from this study demonstrated a significant association of 3 SNPs with osteosarcoma risk. The first (rs1906953; $P = 8.1 \times 10^{-9}$) is located within intron 7 of the glutamate receptor metabotropic 4 (*GRM4*) gene at 6p21.3.³¹ *GRM4* plays a role in cyclic AMP signaling, which has been linked to osteosarcoma in a number of studies,^{40,41} indicating its plausible ability to confer osteosarcoma risk. The locus maps to a DNase I hypersensitivity region in the Encyclopedia of DNA Elements data set, suggesting that it may contain active regulatory elements. The second and third SNPs (rs7591996 and rs10208273; $P = 1.0 \times 10^{-8}$ and 2.9×10^{-7} , respectively) are located in the gene desert at 2p25.2. While neither of these lead SNPs were associated with regulatory elements or transcription factor binding sites in the Encyclopedia of DNA Elements data set, several surrogate SNPs occurred within transcription factor binding sites or altered known regulatory motifs.³¹

Pet dogs develop osteosarcoma that shares many features with the human disease, including tumor histology, gene expression, response to chemotherapy, and risk for pulmonary metastasis.⁴² Accordingly, the dog with osteosarcoma provides a valuable model for the study of cancer-associated genes, drug development, and prognostic markers. A recently published genome-wide association study sought to identify risk loci for osteosarcoma in 3 dog breeds at high risk for osteosarcoma. The study included 286 greyhounds, 135 Rottweilers, and 141 Irish wolfhounds, with relatively equal numbers of cases and controls for each breed. The study identified 33 inherited risk loci accounting for 55–85% of phenotype variance within a breed. The SNP with the strongest association with osteosarcoma development in greyhounds was located 150 kilobases upstream of the *CDKN2A/B* genes, which are known to play a key role in osteosarcoma development and progression (see section III, B, 3, a). The top SNP in Rottweilers and Irish wolfhounds alters an evolutionarily constrained enhancer element that was active in human osteosarcoma cells. Loci among all breeds were enriched for genes with key functions in bone differentiation and development.³²

2. Genetic Syndromes Associated with Osteosarcoma—Increased risk of osteosarcoma is associated with a number of well-defined genetic syndromes: hereditary retinoblastoma (germline mutation of the *Rb* gene), Li-Fraumeni syndrome (germline mutation of the *p53* gene), Bloom syndrome (germline mutation of the *RECQL2* gene), Werner syndrome (germline mutation of the *RECQL3* gene), and Rothmund-Thomson syndrome (germline mutation of the *RECQL4* gene).⁴³ Many of these genes and pathways are commonly altered by somatic mutations in osteosarcoma tumors, although the mechanism of mutation is often distinct from these germline mutations.

B. Somatic Genetic Alterations in Osteosarcoma

As stated above, the rarity of osteosarcoma within the population makes comprehensive genetic and genomic analyses difficult. Numerous studies, often investigating only a subset of common genetic mutations in relatively small patient or cell line cohorts, have been reported. Not surprisingly, such studies make it difficult to confidently assess the frequency and functional consequences of the investigated genetic abnormalities in osteosarcoma. Accordingly, mutation frequencies often are reported as a range identified from multiple publications. Of note, the Therapeutically Applicable Research To Generate Effective Treatments Osteosarcoma Project is currently in progress (<http://ocg.cancer.gov/programs/target/projects/osteosarcoma>). This is a large-scale, multi-institutional collaborative effort to comprehensively identify genetic and epigenetic aberrations in osteosarcoma using a combination of genomic approaches. The results of this study are expected to be published within the next year and should shed new light on the genetic and epigenetic drivers of osteosarcoma. This effort will move osteosarcoma into the postgenomic era, allowing for subsequent interrogations of genetic contributions to osteosarcoma to be completed in silico, a luxury available in the study of other more common cancers but not yet possible for osteosarcoma.

1. Genetic Heterogeneity—As is described in detail below, the mutational landscape of osteosarcoma is highly complex and varies significantly between tumors.⁵ This high degree of intertumor heterogeneity confounds our understanding of the molecular pathogenesis of osteosarcoma and may explain some of the difficulty in identifying therapeutic agents that are likely to improve outcomes for the spectrum of patients with osteosarcoma.

2. Chromosomal Abnormalities—A hallmark of osteosarcoma is chromosomal instability (CIN),^{44,45} a form of genome-wide alteration characterized by a high degree of losses and gains of full chromosomes or chromosomal segments.^{46,47} CIN has been shown to result from a loss of function in cell cycle checkpoint and DNA damage response pathways.^{48,49} As described below, these pathways can be dysregulated in osteosarcoma via both genetic and epigenetic mechanisms. Aberrant maintenance of telomeres through a mechanism known as alternative lengthening of telomeres also has been shown to result in CIN in osteosarcoma.^{50,51}

Unlike many other sarcomas, osteosarcoma lacks a canonical translocation or genetic mutation.^{4,5,52-57} Rather, osteosarcoma is a cancer typified by widespread and heterogeneous abnormalities in chromosomal number and substructure. Osteosarcoma

ploidy can range from haploidy to hexaploidy.^{52,58} While myriad chromosomal losses/gains have been identified, chromosome 1 is most often gained and chromosomes 9, 10, 13, and 17 are most often lost.^{52,58} The most common copy number alterations are deletions of portions of chromosomes 3, 6, 9, 10, 13, 17, and 18 and amplifications of portions of chromosomes 1, 6, 8, and 17. These regions encode a number of tumor suppressors and oncogenes, respectively.⁵

3. Tumor Suppressors

a. Rb Pathway: Rb is a critical regulator of the G₁-to-S cell cycle transition. In the absence of mitogenic stimuli, Rb remains dephosphorylated and binds to E2F family transcription factors, preventing their activation of cell cycle progression. During normal mitosis, this is reversed via Rb phosphorylation by CDK4. Loss-of-function *Rb* mutations remove this cell cycle checkpoint.⁵⁹ The *CDKN2A* locus (also known as *INK4A*) encodes 2 functionally and structurally distinct genes via alternative splicing. The first, *p16^{INK4a}*, is a negative regulator of CDK4. The second, *p14^{ARF}*, is a key regulator of p53 (see below). Loss of *p16^{INK4a}* function alleviates negative regulation of CDK4, resulting in Rb inactivation.⁶⁰ Thus, mutations in the *CDKN2A* gene can phenocopy loss-of-function *Rb* mutations.

Loss-of-function *Rb* mutations occur in up to 70% of osteosarcoma cases;⁶¹⁻⁶⁴ the most common is loss of heterozygosity.^{62,65,66} Other types of *Rb* mutations include structural rearrangements and point mutations.^{61-64,67-69} In one study 70% of patients possessed deletions or rearrangements in the *CDKN2A* gene with the potential to reduce the expression or function of *p16^{INK4a}*.^{57,70-73}

b. p53 Pathway: p53 is a transcription factor that regulates critical genes in DNA damage response, cell cycle progression, and apoptosis pathways.⁷⁴ p53 acts as a tumor suppressor in essentially all tumor types, and its function can be affected by mutations to the gene itself or by mutations to up- or downstream mediators of its activity.⁷⁵ *p14^{ARF}* normally acts to sequester the E3 ubiquitin ligase MDM2 in the nucleolus, preventing it from promoting p53 degradation.⁷⁴ *p14^{ARF}* is expressed from the same *CDKN2A* locus that encodes *p16^{INK4a}* (see above).⁶⁰ Similar to *p16^{INK4a}* in the Rb pathway, loss-of-function mutations in the *p14^{ARF}* gene can phenocopy mutations to *TP53*.⁷⁴

Loss-of-function *TP53* mutations occur in as many as three-fourths of osteosarcoma cases.⁵ These mutations include allelic loss (75–80%), rearrangements (10–20%), and point mutations (20–30%).⁷⁶⁻⁸³ A recent study demonstrated that 9.5% of young patients (<30 years of age) with sporadic osteosarcoma carried either a rare germline *TP53* exonic variant or the canonical Li-Fraumeni mutation, but that these variants are absent from patients who develop osteosarcoma later in life.⁸⁴ As stated above, as many as 70% of osteosarcoma tumors harbor mutations with the potential to affect *p14^{ARF}* expression or function and, therefore, alter p53 function.^{57,70-73}

c. Other Tumor Suppressors: Other tumor suppressors associated with deletions or loss of heterozygosity in osteosarcoma include *APC*, *BUB3*, *FGFR2*, *LSAMP*, *RECQL4*, and *WWOX*.^{65,85-97}

4. Oncogenes

a. Rb Pathway: E2F3 and CDK4, both of which counteract Rb control of cell cycle progression, have been estimated to possess gain-of-function mutations in 60% and 10% of tumors, respectively.^{88,98,99}

b. p53 Pathway: MDM2 is an E3 ubiquitin ligase that acts as a negative regulator of p53 (see above). The *MDM2* gene is amplified in 3–25% of osteosarcoma tumors.^{61,99,102} COPS3 also promotes proteosomal degradation of p53 and is estimated to cause gain-of-function mutations in 20–80% of osteosarcomas.^{92,94,103,106}

c. c-Myc: c-Myc is a key transcription factor that acts as a general amplifier of gene expression, enhancing the transcription of essentially all genes with active promoters in a given cell,^{107,108} and is a well-described oncogene with gained function in most tumor types.¹⁰⁹ *c-Myc* is amplified in 7–67% of osteosarcoma tumors^{88,94,103,110,114} and overexpressed in at least 34% of tumors.^{115,116}

d. Other Oncogenes: Other oncogenes associated with amplifications in osteosarcoma include *CDC5L*, *MAPK7*, *MET*, *PIM1*, *PMP22*, *PRIM1*, *RUNX2*, and *VEGFA*.^{85,88,94,98,104,114,117,125} Collectively, the finding that near ubiquitous alterations in the Rb and p53 pathway function in osteosarcoma through both gain- and loss-of-function mutations indicates that loss of cell cycle control and inappropriate DNA damage response are key drivers of osteosarcoma development. The role that these genetic alterations play in tumor progression and metastasis, however, remains less clear.

C. Mechanisms of Genetic Aberration in Osteosarcoma

As described above, osteosarcoma tumors display a compendium of genetic abnormalities with a high degree of intertumor heterogeneity. While certain genes are commonly altered across tumors, the most common genetic characteristic of osteosarcoma tumors is the remarkable breadth of genetic changes relative to normal tissue. Like the mutations themselves, the mechanisms by which these genetic alterations are acquired are likely to represent a broad spectrum both within and across tumors. Classically defined modes of genetic mutation are known to occur in osteosarcoma. For example, point mutations are likely the result of errors in DNA replication and subsequent proof reading, whereas aneuploidy is the result of errors in chromosomal segregation during cell division.^{126,128} In addition to these well-defined modes of genetic mutation, a novel mechanism of mutation acquisition known as chromothripsis has recently been identified. This term describes a phenomenon by which tens to hundreds of genomic rearrangements occur during cancer development in a one-off cellular crisis. This occurs through reciprocal exchange of genetic material within or between chromosomes. In contrast to the gradual mode of accumulated genetic aberrations in cancer cells acquired through singular mutational events and subsequent Darwinian clonal selection, this model posits “punctuated equilibrium” as the primary mode of tumor evolution. In their landmark paper, Stephens et al.⁶ demonstrated that chromothripsis occurs in at least 2–3% of all cancers and approximately 33% of osteosarcoma tumors.

IV. EPIGENETICS

While cancer has been classically defined as a disease resulting from genetic mutations, a vast and ever-growing body of literature, largely published within the past 15 years, has demonstrated that epigenetic mechanisms are near ubiquitous drivers of tumor development and progression. In this article we refer to *epigenetics* as the study of regulatory mechanisms affecting the expression of DNA templates without altering the sequence of the templates themselves. The most well-described epigenetic mechanisms involved in cancer biology include DNA methylation, histone modification, nucleosome remodeling, and RNA-mediated events.¹²⁹ Importantly, many of these epigenetic processes can be affected by alterations in DNA sequence and vice versa, such that genetic and epigenetic regulation of cellular behavior are inextricable.^{129, 130} The knowledge and technical approaches resulting from the development of this relatively new area of biologic inquiry have provided a new lens through which the biology of cancers, including osteosarcoma, may be investigated.

A. DNA Methylation

Methylation of the 5-carbon on cytosine in cytosine–phosphate–guanine (CpG) dinucleotides represents a key mechanism of epigenetic gene silencing. Roughly 70% of gene promoters contain dense CpG clusters known as CpG islands. Levels of methylation at promoter CpG islands are inversely correlated with gene expression. Gene silencing through promoter hypermethylation represents the most well-described mechanism of epigenetic dysregulation in cancer.^{131, 132}

1. Tumor Suppressors

a. Rb Pathway: While Rb has been shown to be epigenetically silenced by promoter hypermethylation in other cancers,¹³³ there is no strong evidence that epigenetic silencing is a direct mechanism of lost Rb function in osteosarcoma.¹³⁴ However, other members of the Rb pathway are subject to this mode of dysregulation. Specifically, promoter hypermethylation and consequent reduced gene expression have been demonstrated at the *p16^{INK4a}* locus.^{135, 136} Promoter hypermethylation also was detected at the *CREG1* locus, which encodes a gene that acts to enhance p16^{INK4a}-induced cellular senescence in osteosarcoma. Intriguingly, CREG1 expression was restored through treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine (decitabine).¹³⁷

b. p53 Pathway: Similar to Rb, the primary mechanism of direct p53 loss of function in osteosarcoma seems to be genetic.¹³⁴ However, expression levels of multiple members of the p53 pathway are subject to promoter hypermethylation. p14^{ARF} has been shown to be silenced through promoter hypermethylation in both osteosarcoma cell lines and 47% of tumor specimens.^{136, 138} A cell line study suggested that p14^{ARF} expression may be restored by treatment with decitabine.¹³⁸ The same study demonstrated that expression of *p21*, a gene regulated by p53 that blocks G₁-to-S cell cycle progression, was restored by treatment with decitabine, suggesting that this gene also is silenced by DNA hypermethylation.¹³⁹ Another gene target of p53, *GADD45*, which plays a critical role in apoptosis induction in response to DNA damage, possesses a hypermethylated promoter in osteosarcoma cell lines and xenografts.¹³⁹ *GADD45* also has been shown to elicit DNA demethylation in response

to numerous stimuli, indicating that epigenetic silencing of this gene is likely to have pleotropic epigenetic consequences in osteosarcoma.^{11,140,142} In addition, studies combining decitabine treatment with targeted GADD45 knockdown by small interfering RNA have demonstrated that this gene is responsible for apoptosis induction in response to decitabine treatment.¹⁰⁷ HIC1 is a key modulator of p53-dependent responses to DNA damage.¹⁴³ Various studies have shown that 17–77% of osteosarcomas possess hypermethylated *HIC1* promoters and concomitant reductions in HIC1 expression.^{144,145} Inactivation of HIC1 alleviates repression of SIRT1, which subsequently inactivates p53.¹⁴³

These studies indicate that the key Rb and p53 tumor suppressor pathways are subject to both genetic and epigenetic dysregulation in osteosarcoma. In addition, the activity of the p53 pathway affects the epigenomic DNA methylation status of osteosarcoma through the activity of GADD45, demonstrating a connection between DNA damage and altered epigenetic regulation in these cells.

c. Other Tumor Suppressors: Numerous tumor suppressors have been shown to be downregulated through somatic promoter hypermethylation in osteosarcoma cell lines and/or tumor samples. These include *RASSF1A*,^{146–148} *TIMP3*,¹⁴⁸ *MGMT*,¹⁴⁸ *DAPK1*,¹⁴⁸ and *WIF-1*.^{149,150} A subset of these genes has been causally linked to tumor progression in osteosarcoma. Levels of promoter methylation of *RASSF1A*, a gene involved in cell cycle arrest, microtubule stabilization, and apoptosis,¹⁵¹ have been associated with clinical outcomes for patients with osteosarcoma.¹⁵² Like many other genes silenced by promoter hypermethylation, *RASSF1A* levels can be restored by decitabine treatment.¹⁴⁷

2. Oncogenes—There exists to date little to no evidence to suggest that DNA methylation plays a role in oncogene overexpression in osteosarcoma.¹³⁴ There is evidence, however, that oncogene activation may result in downstream epigenetic dysregulation. Activated Ras has been shown to downregulate Fas through DNA methyltransferase (DNMT)–mediated DNA methylation.^{153,154} Downregulation of Fas has been linked to the apoptotic escape of metastatic osteosarcoma cells within the lung microenvironment.^{155–159} Treatment of osteosarcoma cells with ibandronate was shown to inhibit activated Ras and downregulate DNMT expression, resulting in increased levels of Fas expression *in vitro*.¹⁶⁰

B. Histone Modification

The essential units of chromatin structure are nucleosomes, consisting of DNA wrapped around core histone proteins. From each of these proteins protrude amino acid tails that are covalently modified at specific residues to dictate the functional modality of associated DNA elements, both coding and noncoding.¹⁶¹ The patterns of histone tail modifications and resultant genomic functions have been called the “histone code.” As one might predict, there are a large number of “writers,” “readers,” and “erasers” of the histone code, each with a specific role in regulating the biology of the eukaryotic genome.¹⁶² While much about the syntax of this code has been learned in the past several years,^{161,163,164} many of the specifics are still being worked out through experimentation.¹⁶⁵ In parallel, cancer researchers are delineating how alterations in histone modifications contribute to tumor biology.¹⁶⁶ Perhaps

the most well-described influence of histone modifications on cell biology is their effect on the transcriptional output of cells traversing differentiation lineages.^{167,168}

As stated above, the cell of origin and differentiation state of osteosarcoma have yet to be definitively characterized.⁷ It stands to reason that investigations of histone modifications in osteosarcoma cells may yield new insights into this question. While there are limited studies in this area to date, those published have been highly provocative. A number of studies (reviewed by Talluri and Dick¹⁶⁹) demonstrated that Rb plays a role in regulating acetylation and methylation of histones through interactions with specific histone-deacetylating and -methylating complexes. Furthermore, Rb has been shown to broadly regulate chromatin structure through the generation of “senescence-associated heterochromatic foci.”¹⁷⁰ Dysregulation of Rb in osteosarcoma is therefore expected to influence these processes. Interestingly, a recently published study demonstrated that osteosarcoma cells deficient in both Rb and p53 resembled mesenchymal stem cells and were able to differentiate down osteoblastic and adipogenic lineages, whereas osteosarcoma cells deficient in only p53 showed expression patterns concordant with the preosteoblastic state and were unable to differentiate into bone or fat cells.¹⁷¹ These findings suggest that the Rb mutational status of osteosarcoma is a critical determinate of differentiation potential and that this potential is likely influenced by Rb-mediated changes to chromatin.

Another recent study showed that lysine-specific demethylase 1 (LSD1), a histone demethylase, is overexpressed in osteosarcoma tumors and that treatment of osteosarcoma cells with the LSD1 inhibitor tranylcypromine reduced cell growth.¹⁷² LSD1 is known to regulate key processes during cellular development/differentiation,¹⁷³ and its function has been shown to be partially dependent on p53.¹⁷⁴

Finally, it was recently demonstrated that osteosarcoma cells show promoter hypermethylation and concomitant downregulation of a set of 384 genes that also are downregulated in human embryonic stem cells as a result of specific “bivalent” histone modifications linking these genes to pluripotency in human embryonic stem cells.¹⁷⁵ The authors went on to demonstrate that osteosarcoma cells lack histone bivalency at these genes, suggesting that promoter hypermethylation may be a tumor-specific means of dedifferentiation, leading to a transcriptional output that more closely resembles that of pluripotent stem cells than the normal tissue from which the cancer developed.¹⁷⁵

C. Noncoding RNAs

Studies over the past 50 years have demonstrated that a large proportion of genomic DNA is transcribed but not translated into protein (i.e., noncoding).^{176–180} Indeed, a recently published study demonstrated that as much as 75% of the human genome is capable of being transcribed,¹⁸¹ whereas only 1–2% of the genome contains protein-coding genes.¹⁸² Noncoding RNAs are broadly categorized based on their size. Transcripts whose length exceeds 200 base pairs are considered “long,” whereas transcripts below this length threshold are considered “small.” Long noncoding RNAs (lncR-NAs) are defined as any non-protein-coding transcripts over 200 base pairs in length. Many recently published studies have shown lncRNAs to be key regulators of a number of critical biological processes (summarized by Bergmann and Spector¹⁸³). These studies demonstrated that one

primary function of lncRNAs is to regulate gene expression through the formation of ribonucleoprotein complexes that critically influence the chromatin state of the cell.¹⁸⁴ Small noncoding RNAs consist of a large number of subclasses, many of which are key regulators of gene expression. Perhaps the most well studied of these are microRNAs (miRNAs), which act to fine-tune gene expression by binding to messenger RNA transcripts to inhibit translation or induce degradation. The mechanisms of miRNA gene regulation are well described and extensively reviewed elsewhere.¹⁸⁵ Both lncRNAs and miRNAs are critical in tuning gene expression during normal developmental processes. Like other forms of eukaryotic gene control, aberrations in gene regulation by non-coding RNAs can contribute to tumor development and progression.^{186,187} Osteosarcoma is no exception to this rule, and a number of published studies demonstrated the contribution of aberrant noncoding RNAs to osteosarcomagenesis and tumor progression.

1. Long Noncoding RNAs—A recent study⁹¹ showed that chr3q13.31, a region containing the lncRNAs LOC285194 and BC040587, was associated with copy number alteration in 80% of osteosarcomas. The most common mutation type was a deletion that resulted in reduced expression of the associated lncRNAs across osteosarcoma samples. It was further demonstrated that downregulation of LOC285194 promoted the proliferation of normal osteoblasts, supporting the role of this lncRNA as a tumor suppressor in osteosarcoma. LOC285194 knockdown also affected the expression of genes involved in the apoptotic and cell cycle progression pathways, as well as vascular endothelial growth factor receptor 1.⁹¹ A subsequent study¹⁸⁸ showed that LOC285194 is a p53 target and that ectopic expression of LOC285194 inhibits tumor cell growth in vitro and in vivo. Furthermore, this study demonstrated that LOC285194 effects are mediated, at least in part, through its interaction with miR-211. miR-211 expression levels are inversely correlated with those of LOC285194, and miR-211 was shown to promote cell growth.¹⁸⁹ Another study looked at global lncRNA expression changes in 9 osteosarcoma samples and adjacent normal tissue. The authors identified 403 lncRNAs that are consistently upregulated in osteosarcoma and 798 lncRNAs that are consistently downregulated.¹⁸⁹ The biological consequence of altered expression of these lncRNAs remains to be determined.

2. MicroRNAs—miRNAs are a key class of epigenetic regulators that act to post-transcriptionally silence large numbers of genes.¹⁸⁵ Target genes of miRNAs expressed in osteosarcoma include members of signaling pathways that are key to osteosarcoma pathogenesis, including Ras, Wnt, mitogen-activated protein kinase, and Notch.¹³⁴ In addition, miRNAs are capable of broadly affecting epigenetic regulation by silencing DNMTs and, like other transcripts, are themselves subject to epigenetic mechanisms of regulation.¹⁹⁰ A number of studies have demonstrated that dysregulated miRNA expression results in aberrant expression of osteosarcoma genes that play critical roles in tumorigenesis and progression.¹⁹¹⁻¹⁹⁵

miRNAs downregulated in osteosarcoma include miR-16, miR-34, miR-133a, miR-143, miR-199a-3p, miR-335, and miR-340.^{10,189,196-200} Many of these miRNAs are downregulated through epigenetic events in osteosarcoma specifically or in other tumor types.^{196,201,202} miRNAs upregulated in osteosarcoma include miR-20a, miR-29a, miR-140,

and miR-181.^{193,195,203,205} In addition to targeting key genes in osteosarcoma signaling pathways, as described above, a number of miRNAs upregulated in osteosarcoma affect epigenetic regulatory genes. miR-140 has been shown to target histone deacetylase 4.²⁰⁵ miR-20a is thought to be responsible for epigenetically mediated downregulation of Fas.²⁰³ Like other oncogenes described above, there exists little to no evidence demonstrating epigenetic changes as a key driver of miRNA overexpression.¹³⁴ However, this is likely explained by the fact that most epigenetic studies of osteosarcoma completed to date have focused on DNA methylation, which provides only the opportunity to detect hypomethylation as a means of gene activation and would not detect other well-described mechanisms of epigenetically mediated overexpression.

V. POTENTIAL NEW TARGETS FOR THERAPY

In osteosarcoma and other solid tumors with high rates of metastases, therapeutic targets that may most improve patient outcomes have been recognized to be those that target metastatic progression and, as such, may not have substantial activity on measurable primary tumors. Furthermore, the fact that osteosarcoma lesions are associated with a rich bone stroma that may not immediately regress concurrent with a tumor response to therapy complicates the conventional use of tumor response to identify agents that may be active in osteosarcoma. For both of these reasons, drugs with potential clinical efficacy may reasonably fail to show activity in standard phase II clinical trials, which rely on shrinkage of the primary tumor as the key metric of therapeutic response. In light of this, the osteosarcoma drug development community has recently outlined the types of preclinical data that should be prioritized as a novel therapeutic agent is considered for inclusion in the treatment of patients with osteosarcoma as a means to prevent metastatic progression, realizing that response data in human patients may not be available.²⁰⁶ To help assess the potential clinical utility of novel therapeutic agents, an important model/tool is provided by pet dogs that develop osteosarcoma. Indeed, studies of dogs with osteosarcoma are now underway to best define the activity of agents with the greatest promise to improve outcomes for patients. An additional resource available to the osteosarcoma preclinical/clinical research community is the data generated by the Pediatric Preclinical Testing Program, a program to systematically evaluate new agents against childhood leukemia and solid-tumor models (including osteosarcoma). Pediatric Preclinical Testing Program data are publicly available online (<http://pptp.nchresearch.org/>).

Table 1 presents a list of therapeutic agents that may be reasonably considered to improve treatment outcomes for patients with osteosarcoma. These agents were selected for inclusion based on their specificity for targeting the genetic and epigenetic alterations identified in osteosarcoma and presented in this article, for targeting other key osteosarcoma pathways, or for their promise in preclinical and clinical studies. For each agent listed, a subjective measure of the strength of evidence (based on our assessment) is included.

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ABBREVIATIONS

CIN	chromosomal instability
CpG	cytosine-phosphate-guanine
DNMT	DNA methyltransferase
DNMTi	DNA methyltransferase inhibitor
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
hESCs	human embryonic stem cells
lncRNA	long non-coding RNA
LSD1	lysine-specific demethylase 1
miRNA	microRNA
mTOR	mammalian target of rapamycin
Rb	retinoblastoma
SNP	single nucleotide polymorphism
VEGF	vascular endothelial growth factor

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FIG. 1. Identified molecular alterations leading to metastatic traits of osteosarcoma cells. This image depicts the genetic, epigenetic, and other molecular biological processes whose defects have been associated with metastatic traits of osteosarcoma cells. lncRNA, long noncoding RNA; mRNA, messenger RNA; miRNA, microRNA.

TABLE 1

Candidate Osteosarcoma Therapeutic Agents

Agent	Target(s)	Mechanism of Action	Preclinical/Clinical Rationale	Strength of Evidence *
Chemotherapeutics and small-molecule inhibitors				
Gemcitabine, aerosolized	Chemotherapeutic agent; Fas	Pyrimidine antimetabolite; upregulates Fas expression	Inhibited metastasis in osteosarcoma xenograft models ²⁰⁷ ; effect abolished in FasL-deficient mice ¹⁵⁷	Medium-high
RG7388	MDM2	Small-molecule inhibitor of p53–MDM2 interaction	Evidence for dysregulation of p53/Mdm2 in most osteosarcoma (see text); inhibited osteosarcoma tumor growth in xenograft models ²⁰⁸	Medium-high
PF-2341066	Met	Small-molecule, ATP-competitive Met inhibitor	Evidence for overexpression in osteosarcoma tissues; overexpression linked to metastasis biology; reduced primary tumor growth and metastasis in xenograft models ²⁰⁹	Medium-high
NSC305787; NSC668394	Ezrin	Protein–protein interaction inhibitors, specific kinase inhibitors	Expression associated with a less favorable outcome ²¹⁰ ; knockdown inhibited metastasis in xenograft models ²¹⁰ ; small-molecule inhibitor reduced invasive phenotype in vitro ²¹¹	Medium
Vismodegib (GDC-0449)	Hedgehog (HH) pathway	Smoothened receptor (SMO) antagonist	Known role in stem cell differentiation during normal bone development; known role in metastasis in other cancers ²¹² ; pathway inhibition inhibits tumor growth in xenograft model ²¹³ ; FDA approved for treatment of other cancers ²¹⁴	Medium
Saracatinib (AZD0530)	Src	Selective Src kinase inhibitor	Reduced cell motility in vitro, no reduction of metastasis in mouse models ²¹⁵ ; phase II.5 clinical trial underway (www.clinicaltrials.gov identifier NCT00752206)	Medium-low
Rapamycin	mTOR	Small-molecule inhibitor	Signaling pathway active in osteosarcoma tissues ²¹⁶ ; expression correlated with metastasis and survival ²¹⁶ ; currently in clinical trials in dogs (COTC020); prevented metastasis in xenograft	Medium

Agent	Target(s)	Mechanism of Action	Preclinical/Clinical Rationale	Strength of Evidence *
			models ²¹⁷	
Immune modulators and antibody conjugates				
hu14.18K322A	GD2	Humanized anti-GD2 antibody	Ubiquitously expressed in osteosarcoma cell lines and tissues ²¹⁸ ; currently being tested in phase I clinical trials in osteosarcoma (www.clinicaltrials.gov/identifer/NCT00743496)	Medium-low
ADX31-164	Her2/neu	Vaccine	Expression in osteosarcoma tumors is associated with poor survival outcomes ^{219,220} ; targeted immunotherapy reduced tumor-initiating cells ²²¹ ; currently in dog clinical trials (www.petcancerinformation.com)	Medium
Glembatumumab vedotin (CDX-011)	GPNMB	Antibody–auristatin conjugate	Variably expressed on surface of osteosarcoma xenografts ²²² ; significant improvement in event-free survival in osteosarcoma xenograft models ²²²	Medium
Epigenetic modulators				
5-aza-CdR (decitabine)	CREG1, p14ARF, p21, RASSF1	DNMTi	Numerous genes associated with promoter hypermethylation in osteosarcoma (see text); phase I clinical trials completed ²²² and ongoing (www.clinicaltrials.gov/identifer/NCT01241162)	Medium-low
Ibandronate	Ras, DNMT, Fas	Bisphosphonate; upregulates Fas	Inhibited Ras function and downregulated DNMT, leading to increased Fas expression ¹⁶⁰ ; induced apoptosis in vitro ¹⁶⁰	Low
Zolendronate	Small GTPases	Bisphosphonate; downregulates VEGF	Suppressed lung metastasis and prolonged overall survival in mouse models ^{224,225} ; phase I clinical trial completed ²²⁶	Medium-high
Tranylcypromine	LSD1	Forms adduct with inactive region of LSD1	Expressed in osteosarcoma tissues ¹⁷² ; reduced osteosarcoma growth in vitro ¹⁷²	Low
Pracinotat (SB939)	HDAC	HDACi	Phase I trial completed ²²⁷	Medium-low
Erinostat (MS-275)	HDAC, Fas	HDACi; Fas upregulation	Upregulates Fas expression in Fas-metastatic osteosarcoma	Medium

Agent	Target(s)	Mechanism of Action	Preclinical/Clinical Rationale	Strength of Evidence *
			cells ²²⁸ ; caused regression of metastasis in xenograft models through upregulation Fas expression in Fas- cells ²²⁸	
Valproic acid	HDAC	HDACi	Inhibited growth in vitro and in xenograph metastasis models in combination with doxorubicin ²²⁹ ; phase I clinical trials ongoing (www.clinicaltrials.gov identifiers NCT01106872; NCT01010958)	Medium-high

* Strength of evidence assessment represents a subjective measure on a scale of “low” to “high,” as judged by the authors based on efficacy in preventing or reversing metastatic progression in preclinical models as well as safety and therapeutic efficacy in dog and human clinical trials.