Biology of Osteogenic Sarcoma

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ABSTRACT

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. Despite significant clinical improvements over the past several decades through the use of combination chemotherapy and surgery, patients with metastatic or recurrent disease continue to have a very poor prognosis. Therefore, there is a continued need to study and understand the basic biology of osteosarcoma in order to devise more targeted and rational therapeutic strategies and ultimately to improve survival for these patients. This article reviews several aspects of osteosarcoma biology where data exist to suggest that specific pathways may play a role in the pathogenesis of this tumor. These areas include host genetic predispositions, tumor cytogenetics, molecular genetics (including the Rb, p53, RECQ helicase, and telomere pathways), and metastatic factors (ezrin, annexin 2, chemokine receptor 4, Fas/FasL pathways) that may contribute to both the initiation and the progression of tumor formation. Understanding the mechanisms of and interactions between the various molecular pathways that play a role in osteosarcoma pathogenesis may eventually lead to a more rational strategy for devising therapies targeted specifically toward these pathways. *(Cancer J 2005;11:294–305)*

KEY WORDS

Osteosarcoma, p53, RB, RECQ helicase, telomere maintenance, chromosomal instability

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BIOLOGY OF OSTEOGENIC SARCOMA

Osteosarcoma is a primary malignant tumor of bone[1] that accounts for approximately 5% of childhood cancers overall. Only about 400 cases are diagnosed each year in the United States.[2] Despite its rarity, osteosarcoma is the most common primary malignancy of bone in children and adolescents and is the fifth most common malignancy among young adults aged 15–19 years.[3] Although survival of patients with osteosarcoma has improved dramatically over the past 30 years, largely as a result of chemotherapeutic advances, patients who present with metastatic disease and those who have disease recurrence continue to have a very poor prognosis (less than 20% long-term survival).[4,5] Therefore, there is a continued and important need for ongoing studies of the basic biology and underlying pathogenesis of osteosarcoma in order to devise more targeted and rational therapeutic strategies and ultimately to improve survival for these patients. This article reviews what is currently known about some aspects of the biology of osteosarcoma with regard to host genetic predispositions, tumor cytogenetic abnormalities, molecular genetic aberrations, and metastatic factors.

GENETIC PREDISPOSITION TO OSTEOSARCOMA

Most osteosarcomas are sporadic, whereas inherited predisposition accounts for a small number of cases. Genetic conditions with a known predisposition to osteosarcoma include hereditary retinoblastoma, Li-Fraumeni syndrome, Rothmund-Thomson syndrome, and Werner syndrome. These genetic syndromes are associated with constitutional mutations in genes that are involved in cell cycle control or DNA metabolism and may provide important clues to the pathogenesis of sporadic osteosarcomas.

Hereditary Retinoblastoma

Retinoblastoma is a malignant tumor of the retina that involves mutations in the RB gene.[6] These tumors are often present at birth and are almost entirely

restricted to early childhood. They may be unilateral or bilateral and may be of the sporadic or the hereditary type. Approximately 40% of patients have the heritable form of retinoblastoma and are heterozygous for germline-inactivating mutations in the *RB* gene, which is critical in cell cycle control. This group of patients has an increased risk of developing second primary tumors, 60% of which are sarcomas, and about half of these are osteosarcomas.[7-9] Patients with the sporadic form of retinoblastoma are at a much lower risk for developing osteosarcoma. Given the increased incidence of both primary and radiation-related osteosarcomas in patients with germline mutations in RB in combination with the fact that most sporadic osteosarcoma tumors have alterations in the RB pathway, this gene is likely to be central to the molecular pathogenesis of osteosarcoma.

Li-Fraumeni Syndrome

Li-Fraumeni syndrome is a familial cancer syndrome in which affected family members display a spectrum of cancers, including breast, soft tissue, adrenocortical, and brain tumors; leukemias; and osteosarcomas.[10] Many of these patients carry germline-inactivating mutations in the *p53* tumor suppressor gene, which is involved in cell cycle regulation as well as maintenance of genome integrity.[11] Despite this important association, the number of osteosarcomas that are attributable to Li-Fraumeni syndrome is small.[12,13] In one series of 235 unselected children with osteosarcoma, only 3% carried constitutional germline mutations in *p53*.[14] However, *p53* alterations are found very frequently in sporadic osteosarcoma, suggesting a critical role of this gene in the pathogenesis of osteosarcoma.

RECQ Helicase Disorders

The RECQ helicases are a family of conserved proteins that share common amino acid sequence motifs in the DNA helicase domain. There are five members of this family in humans, three of which are associated with cancer predisposition syndromes. One of these, Rothmund-Thomson syndrome (RTS), is an autosomal recessive condition characterized by distinctive skin findings (atrophy, telangiectasias, pigmentation), sparse hair, cataracts, small stature, skeletal anomalies, and a significantly increased risk for osteosarcoma. In one cohort of 41 patients with RTS, 13 (32%) developed osteosarcoma.[15] Clinically, these tumors tend to develop at a younger age than seen in the general population (median age, 9 years). Loss of function mutations in the *RECQL4* gene have been identified in approximately two thirds of patients with RTS, and the presence of mutations is correlated with the risk for developing osteosarcoma. In one series of 33 patients with RTS, none of the 10 patients without a truncating mutation in this gene developed osteosarcoma, whereas among the 23 patients with truncating mutations, the incidence of osteosarcoma was five cases per year, with 230 person-years of observation.[16] Two other members of the *RECQ* gene family are mutated in Bloom syndrome and Werner syndrome, two diseases with overlapping clinical features, including a predisposition to developing cancers.[17] Patients with Bloom syndrome have mutations in the *BLM* gene and are predisposed to virtually all the types of cancers seen in the general population, except at a much younger age and at a higher frequency.[18] Patients with Werner syndrome have mutations in the *WRN* gene and are predisposed to developing osteosarcomas, although not at as high a rate or with such specificity as in RTS, and they are also at increased risk for other soft tissue sarcomas, thyroid cancers, and melanomas.[19] Recently, mutations in *RECQL4* have also been identified in patients with the RAPADILINO syndrome, which is characterized by small stature, radial ray defects, joint and knee problems, and gastrointestinal disturbance. One of 14 patients developed osteosarcoma, and whether this disorder also represents an osteosarcoma predisposition syndrome remains to be seen.[20] Of all the osteosarcoma predisposition syndromes, RTS appears to have the highest and most specific risk for this particular tumor.

Paget's Disease

Although a disease of older adults, Paget's disease is also associated with development of osteosarcoma; in fact, osteosarcomas in patients older than 40 years are almost always associated with Paget's disease.[21] Paget's disease is a condition characterized by excessive focal bone resorption with subsequent increased bone formation and eventual replacement of normal bone marrow by vascular and fibrous tissue. Approximately 2% of patients with Paget's disease develop osteosarcoma.[1] Both genetic and environmental factors have been implicated in the pathogenesis of this disorder. Some patients with Paget's disease carry mutations in the *SQSTM1* gene on chromosome 5q35 that encodes sequestosome 1 (also known as p62), which is a ubiquitin-binding protein involved in the interleukin-1, tumor necrosis factor, and RANKL signaling pathways that are important in osteoclastogenesis.[22] Mutations in *SQSTM1* have been detected in both familial and nonfamilial cases of Paget's disease.[23] About 30%

of patients studied with hereditary Paget's disease carried mutations in this gene.[23] However, no *RANKL* mutations have been detected in osteosarcoma samples thus far.[21] Although *SQSTM1* is the gene most frequently linked to Paget's disease, several other susceptibility loci have also been identified.[24] Whether these genes play a role in the pathogenesis of pediatric osteosarcomas remains to be seen.

CYTOGENETIC AND MOLECULAR ABNORMALITIES IN OSTEOSARCOMA

The specific nature of the aberration (or aberrations) that lead to tumorigenesis remains elusive in osteosarcoma. In contrast to other sarcomas, such as synovial sarcoma, alveolar rhabdomyosarcoma, and Ewing's sarcoma, in which recurrent chromosomal translocations are characteristic, osteosarcomas do not have any specific translocations or other molecular genetic abnormalities that can serve as diagnostic or tumor-specific markers of disease. Most osteosarcomas display complex numerical and structural chromosomal abnormalities with significant cell-to-cell variation and heterogeneity.[25,26] Similarly, these tumors can have a variety of nonspecific alterations that involve inactivation of tumor suppressor genes, such as *p53* and *Rb*, or overexpression of oncogenes, such as myc and *Her-2*. Dissecting out these various chromosomal and molecular aberrations and understanding the relative contributions of the different molecular pathways remains a challenge in osteosarcoma biology but will likely provide important information regarding the underlying pathogenesis of this disease.

Cytogenetic Abnormalities

A multitude of cytogenetic abnormalities have been detected in osteosarcoma tumors. Early studies using conventional cytogenetic techniques revealed various clonal chromosomal abnormalities, numerical abnormalities (+1, −9, −10, −13, −17), partial or complete loss of 6q, and rearrangement involving regions 1p11-13 (15%), 1q10-12 (19%), 1q21-22 (14%), 11p15 (17%), 12p13 (15%), 17p12-13 (14%), 19q13 (17%), and 22q11-13 (15%).[25,26] More recent studies have combined several molecular cytogenetic techniques to assess chromosomal changes in osteosarcoma. A recent study by Lau et al[27] used a combination of modalities, including comparative genomic hybridization, spectral karyotyping, and fluorescence in situ hybridization to analyze a panel of 25 osteosarcoma specimens to provide a more detailed assessment of the complex cytogenetic aberrations in osteosarcoma. The most frequently detected amplifications were of chromosomal bands 6p12-p21 (28%), 17p11.2 (32%), and 12q13-q14 (8%). Several other recurrent chromosomal losses (2q, 3p, 9, 10p, 12q, 13q, 14q, 15q, 16, 17p, and 18q) and chromosomal gains (Xp, Xq, 5q, 6p, 8q, 17p, and 20q) were also identified, as well as several recurrent breakpoint clusters and nonrecurrent reciprocal translocations. This and other previous studies[28-32] highlight the complexity and the instability of the genetic makeup of osteosarcomas. Using this sort of combined refined approach to identify specific chromosomal regions perturbed in osteosarcoma will allow future detailed investigation of the affected regions for potential candidate genes that may play a role in the pathogenesis of osteosarcoma.

Tumor Suppressor Pathways

The highest frequency of loss of heterozygosity (implying loss of a putative tumor suppressor gene) in osteosarcoma is reported for chromosomes 3q, 13q (the location of the retinoblastoma gene), 17p (the location of the *p53* gene), and 18q (the chromosomal region that has been linked to osteosarcomas arising in the setting of Paget's disease).[33-35] Several other regions of recurrent chromosomal losses or rearrangements may also potentially harbor other tumor suppressor loci, but candidate genes have yet to be identified in these areas.[36,37] Alterations in the most well-characterized tumor suppressor pathways involving RB and p53 in osteosarcoma are discussed in the following sections.

The RB Pathway Patients with germline mutations in the *RB* gene are at an increased risk of developing osteosarcoma. The RB protein is a major regulator of cell cycle progression from G_1 to S phase[37] and thus may have an antiproliferative role in terms of restricting tumor growth. Other members in this pathway include cyclin-dependent kinase-4 and 6 (CDK4/6), cyclin D1, and $p16^{INK4a}$. Therefore, alterations in any one of the genes encoding these proteins, or a combination of mutations, may play a role in tumorigenesis. $p16^{INK4a}$ inhibits CDK4/6, which, in complex with cyclin D1, is responsible for the phosphorylation of RB, thereby blocking transition from G_1 to S. In its dephosphorylated state, RB is bound by E2F, a transcription factor that promotes DNA synthesis and cell cycle transition from G_1 to S.[38] Thus, both $p16^{INK4a}$ and RB have tumor suppressor roles, and mutations in either could result in loss of checkpoint function. Alternatively, overexpression or amplification of cyclin D1 or CDK4/6 could also result in functional inactivation of the Rb signaling pathway, thus leading to tumorigenesis. Alterations in these proteins have all been described in sporadic osteosarcoma tumors. In

addition to this checkpoint function, RB also plays a role in orchestrating other phases of the cell cycle and has multiple additional cellular roles, including mediating differentiation signals and modulating chromosome segregation during mitosis, which contributes to the overall maintenance of genomic integrity.[39] Disruption of any of these functional pathways of RB could potentially contribute to increased proliferation or genomic instability leading to tumorigenesis.

RB Alterations in the RB pathway are frequently found in osteosarcoma tumor samples and appear to be critical in the pathogenesis of these tumors. Approximately 70% of primary osteosarcoma tumors have alterations located on chromosome 13q14 in the RB gene itself. These include gross structural rearrangements, complete deletion, and point mutations.[40-42] Wadayama et al. conducted a comprehensive mutation analysis of 63 osteosarcoma samples and detected loss of heterozygosity at 13q in approximately 60% of tumors, gross structural rearrangements in about 30%, and point mutations in less than 10%.[40]

p16^{INK4a} The cyclin-dependent kinase inhibitor p16^{INK4a} has also been found to be inactivated in about 10% of osteosarcomas.[43-45] The p16^{INK4a} protein is encoded by the *INK4a/ARF* locus (also called *CDKN2a*) located on chromosome 9p21. *INK4a* encodes p16^{INK4a} and *ARF* encodes p14^{ARF}, a protein that regulates p53 and is also mutated in osteosarcomas. These two different proteins are formed through bicistronic transcription and differential splicing into alternative reading frames and therefore are not isoforms and share no amino acid homology.[38] However, they both play dual but nonoverlapping roles in mediating senescence and suppressing malignant growth. Most of the alterations involving *INK4a* or *p16^{INK4a}* represent deletions rather than point mutations.[44,45] Mutations have also been detected in osteosarcomas in the related *p19^{INK4a,46}* which has structural and functional similarity to *p16^{INK4a}*. Alterations (gene rearrangements) were detected in 7% (5/67) of osteosarcomas but in none of the other 16 types of sarcomas or 34 non–small cell lung cancers studied. This is in contrast to p16^{INK4a}, which is found to be mutated in a variety of tumor types, including melanoma, glioblastoma, pancreatic adenocarcinoma, non–small cell lung cancer, bladder carcinoma, and oropharyngeal cancer.[38,47]

CDK4 and Cyclin D1 Amplification of the chromosomal region 12q13-15 occurs in approximately 10% of osteosarcomas.[31] This region contains CDK4, which along with cyclin D1 is responsible for RB phosphorylation and loss of checkpoint function. This region also contains MDM2, which is a regulator of p53. In one study of 87 osteosarcoma samples, CDK4 was amplified in 6/17 samples, and none of these amplifications occurred in tumors in which *INK4a* was either deleted (4/55) or rearranged (1/55).[45] Cyclin D1 has also been shown to be amplified in approximately 4% of cases of osteosarcoma.[43,45]

The p53 Pathway The *p53* gene, located on chromosome 17p13.1, is the most commonly mutated gene in human cancers.[48-50] As previously discussed, patients with Li-Fraumeni syndrome who carry constitutional mutations in *p53* are at high risk for developing a variety of cancers, including osteosarcoma. Similarly, in mouse models of Li-Fraumeni syndrome, up to two thirds of mice with one null or mutant copy of *p53* develop osteosarcoma.[51,52] The p53 protein has multiple roles as a central regulator of cellular response to stress.[53] It regulates many different downstream target genes, including those involved in cell cycle control and apoptosis. The p53 pathway is linked to the RB pathway in its regulation of the cell cycle.[54] On stabilization and activation, p53 directs the sequence-specific transcriptional activation of p21, a cyclin-dependent kinase inhibitor that can inhibit the activities of cyclin D-CDK4/6 or cyclin E-CDK2, thereby decreasing RB phosphorylation and arresting cells in G_1. One of the mechanisms of p53 regulation is through ARF and MDM2. *ARF* is a gene located at the same locus as INK4a on chromosome 9p1 and encodes p14^{ARF}. p14^{ARF} in turn binds to and inhibits *MDM2*, which possesses E3 ubiquitin ligase activity that targets p53 for proteosomal degradation. Therefore, through inhibition of MDM2, ARF stabilizes p53 and leads to growth arrest or apoptosis, depending on the cellular context. Thus, similar to the situation with the RB pathways in osteosarcoma, both ARF and p53 have tumor suppressor roles, and mutations in either could result in loss of checkpoint function. Alternatively, overexpression or amplification of the *MDM2* gene could also result in functional inactivation of the p53 signaling pathway. These alterations have all been detected in sporadic osteosarcoma tumors. In addition to the G_1/S checkpoint function, p53 plays a major role in DNA damage response and induction of apoptosis, as well as a role in DNA repair and recombination, functions that if perturbed may also contribute to tumorigenesis.[55] Wild-type p53 also appears to play a role in the normal development and physiology of bone[56] because *p53*-null mice display failure of skull growth and delayed longitudinal bone growth in utero.[57] Bone cell lysates from *p53*-null mice also fail to activate normal apoptotic pathways.[58]

p53 Inactivating mutations of the *p53* gene occur in approximately 50% of all sporadic cancers. Among os-

teosarcomas, the overall frequency of *p53* mutations ranges from 15% to 30%, depending on the detection methods used.[59] The types of alterations include point mutations (~20%–30%), gross gene rearrangements (~10%–20%), and allelic loss (~75%–80%).[60,61] Most point mutations in osteosarcoma are represented by missense mutations occurring in exons 5–8, which is in the DNA binding domain of the gene.[59] A few groups have looked at whether *p53* status in osteosarcoma tumors correlates with patient outcome and metastatic disease status. Wunder et al[62] conducted a prospective study of 168 patients with nonmetastatic osteosarcoma to determine where there was an association between *p53* status and risk of systemic relapse. They detected *p53* mutations in 19% of tumor samples, most of which were missense mutations, and of the tumors with mutations, 42% had evidence of loss of heterozygosity. However, the investigators did not detect an association between *p53* mutation status and risk of relapse. Similarly, Gokgoz et al[59] examined whether there was a difference in *p53* mutation status between nonmetastatic and metastatic tumor samples, and they were not able to detect a difference, coming to the conclusion that *p53* events were likely not to be a late event in tumor progression.

MDM2 *MDM2* (mouse double-minute 2 protein, also called *HDM2* for the human ortholog), located on chromosome 12q13, encodes a protein that binds p53 and blocks transcriptional activation of downstream genes and also targets p53 itself for degradation. Thus, *MDM2* amplification and overexpression results in functional suppression of p53, even in the presence of wild-type p53 protein. The 12q13 region where *MDM2* (as well as *CDK4*) resides is amplified in 10%–20% of osteosarcomas.[63-65] *MDM2* was found by Southern blotting to be amplified in 5/75 (7%) osteosarcoma samples in one study[63] and in 4/28 samples (14%) in another study, all of which represented recurrent or metastatic disease.[66] Overholtzer et al[67] examined 34 osteosarcoma specimens to determine whether there was a correlation between *p53* (or *HDM2*) status and genomic instability in the tumors. Thirty-eight percent of the tumors were found to have *p53* missense mutations, and 15% of tumors had a greater than threefold amplification of *HDM2* over control subjects. Using comparative genomic hybridization techniques to identify chromosomal copy number changes as a measure of genomic instability, the investigators were able to detect frequent deletions and amplifications. The most recurrent losses were of chromosomal regions 18q21q23 (41%), 6q16 (38%), and 10q22q26 (35%). The most frequently gained or amplified chromosomal regions were at 17p11.2 and 6p12 (47%), 1p32p36 (44%), and 8q24 (44%). They found that *p53* mutant tumors displayed high levels of genomic instability, whereas *HDM2* amplified tumors did not, suggesting that inactivation of *p53* directly by mutation may have different cellular consequences than indirect amplification of *HDM2* in terms of maintaining genomic stability.

p14^{ARF} The p14^{ARF} protein is encoded (along with p16^{INK4a}) by the *INK4a/ARF* locus located on chromosome 9p21. p14 regulates p53 function by binding MDM2 and sequestering it in the nucleolus, thereby preventing the shuttling of p53 to the cytoplasm for proteasome degradation. *INK4a/ARF* has been found to be deleted in approximately 10% of osteosarcoma tumors.[68]

The RECQ Helicase Pathways RECQ helicases are DNA unwinding enzymes that are involved in many basic cellular processes, including DNA replication, transcription, and repair and are believed to be important in maintaining genomic integrity. Patients with constitutional mutations in three of the five human RECQ helicases (BLM, WRN, and RECQL4) are at increased risk for developing a variety of cancers; therefore, these helicases are believed to play a role in tumor suppression. They are considered to be "caretakers" of the genome, that is, proteins that do not directly regulate tumorigenesis (e.g., RB or p53, which are considered gatekeepers of the genome), but instead influence the stability of the genome and therefore affect the rate of accumulation of genetic alterations that ultimately result in tumor formation.[69] Much recent work has investigated the functions of the RECQ helicases, in particular, the roles they play in cancer and aging.[70] There is a growing body of biochemical and genetic evidence to suggest that the BLM and WRN RECQ helicases facilitate the process of DNA replication by removing potential roadblocks in the form of complex DNA structures, and that they are also involved in the reinitiation of DNA replication at the sites of replication fork arrest or collapse.[69,70] Less is known about the function of the RECQL4 protein or the exact role that it plays in osteosarcoma pathogenesis. The very high and specific risk of osteosarcomas in patients with RTS carrying constitutional mutations in *RECQL4* suggests that this gene might play a specific role in the pathogenesis of osteosarcoma.[16] *RECQL4* is located on chromosome 8q24.3, which has been shown to be a frequent site of chromosomal gain in comparative genomic hybridization analyses of osteosarcomas.[30,32] To investigate the frequency of somatic mutations in osteosarcomas in the general population, Nishijo et al[71] examined 71 sporadic osteosarcoma tumors from a

Japanese population for mutations in *RECQL4* by DNA sequencing of all 21 exons and 16 short introns; the investigators found only two base changes and a six base pair in frame deletion, which did not disrupt the reading frame. The frequency of each allele was not significantly different between osteosarcoma and control populations, leading to the conclusion that the *RECQL4* gene is unlikely to be a major contributor to the development of sporadic osteosarcomas. However, given the common theme of cancer predisposition among patients with RECQ deficiencies (Bloom, Werner, and Rothmund-Thomson syndromes), it is possible that even in the absence of direct gene mutations within tumors, the pathways in which these helicases participate play a role in modulating the development of osteosarcoma and other cancers in the general population.

Oncogene Overexpression

Although many proto-oncogenes are overexpressed in osteosarcomas, the data regarding the exact role of this overexpression in the pathogenesis of osteosarcoma have not been definitive.[35] Some of the proto-oncogenes that have been investigated include *c-myc, c-fos, Her-2, MET, SAS,* and *GLI* . Both *c-myc* and *c-fos* have been shown to be overexpressed in osteosarcoma samples.[72,73] c-fos is part of the transcription factor complex AP-1 and has been shown to play a role in modulating both osteoclast differentiation and osteoblast transformation[74] and may play a more specific role in osteosarcoma. Mice overexpressing *c-fos* are prone to developing osteosarcomas.[75,76] In one study of human osteosarcoma tumors from 38 patients,[77] co-overexpression of both *c-myc* and *c-fos* was detected at a significantly higher rate in patients with a lower rate of disease-free survival than in patients with overexpression of only one of the two transcription factors. *c-fos* is transcriptionally modulated by parathyroid hormone and posttranslationally regulated through phosphorylation by *RSK2*, which has been shown to be mutated in the human disease Coffin-Lowry syndrome, which is marked by progressive skeletal abnormalities.[78,79] In a recent study of *c-fos* transgenic mice lacking *RSK2*, David et al[79] showed that RSK2-dependent stabilization of c-fos is essential for osteosarcoma formation in these mice, suggesting that *RSK2* may also play a role in human osteosarcomas.[79]

Her-2 is a cellular proto-oncogene that encodes the human epidermal growth factor receptor 2 (Her-2), a transmembrane receptor tyrosine kinase. Overexpression of *Her-2* is clearly associated with a metastatic phenotype in breast cancers for which trastuzumab, a monoclonal antibody against the Her-2 receptor, is clinically available.[80] Overexpression of *Her-2* has also been reported in some osteosarcomas. However, the studies have been controversial regarding the clinical significance of overexpression of *Her-2* in osteosarcoma as well as the interpretation of the overexpression itself, due to differences in methodology.[81] *Her-2* overexpression has been found to be common by some groups, who believe it to correlate with poor clinical outcomes,[82-84] whereas other groups have found overexpression to be very rare or absent.[85-88] To address some of these historical issues regarding technical and methodological discrepancies, Fellenberg et al[89] used a laser-microdissection technique in combination with real-time reverse transcriptase-polymerase chain reaction to examine archived tumor biopsy samples, and they were able to optimize these techniques to demonstrate *Her-2* expression and to correlate *Her-2* expression with histologic response to chemotherapy.[89] Larger, prospective studies incorporating standardized methodologies may eventually reveal whether overexpression of *Her-2* plays a role in the clinical outcomes of patients with osteosarcoma and whether use of trastuzumab will play a role in targeted therapy for osteosarcoma tumors that overexpress *Her-2*.

Telomere Maintenance

Recent work has emerged regarding the maintenance of telomeres in osteosarcoma. Telomeres are the repetitive DNA sequences at the ends of chromosomes that shorten with every cell cycle division in human cells.[90] Gradual shortening of telomeres eventually leads to cellular senescence. The ability to lengthen telomeres is an effective mechanism for tumors to evade senescence and therefore to promote growth and perpetuation.[91] There are two main telomere maintenance mechanisms (TMM) by which tumors can prevent critical shortening of telomeres: through an enzyme called telomerase, which adds telomeric repeats and prevents shortening of telomeres, and/or through an alternative lengthening of telomeres (ALT) pathway that is believed to be recombination based.[92-94] Unlike many cancers that predominantly utilize telomerase,[95,96] more than 50% of osteosarcomas utilize the ALT mechanism.[97,98] Recent work has attempted to determine whether there is a correlation between TMM status in osteosarcoma tumors and outcomes in patients. Previous work in mouse models or in vitro systems have shown that an aggressive phenotype of tumors is dependent on the presence of telomerase,[96,99,100] even in tumors with alternative telomere lengthening mechanisms. Ulaner et al[101] examined TMM in primary osteosarcoma samples from 60 patients and found the following distribution of

TMM: telomerase–/ALT+ in 21 patients, telomerase+/ALT– in 11 patients, telomerase+/ALT+ in 17 patients, and telomerase–/ALT– in 11 patients.[101] The telomerase–/ALT– tumors were associated with better overall survival than the tumors with only one TMM, but the presence of telomerase activity was not predictive of survival. This is in contrast to a study by Sanders et al,[98] who evaluated osteosarcoma primary tumors from 44 patients. They found again that ALT was the primary TMM (70%), compared with telomerase alone (using *TERT* expression) (18%), and that some tumors used both TMM (14%), whereas others had neither TMM (2%). However, in their study looking at clinical outcomes in relation to TMM, Sanders et al found that patients with TERT+/ALT+ tumors had outcomes superior to those in patients whose tumors were TERT+/ALT–, leading the authors to suggest the possibility that the ALT phenotype might somehow act to mitigate the aggressiveness conferred by *TERT* expression. Future work in this area using larger, prospective cohorts will help clarify some of these issues regarding the utility of TMM status as a prognostic marker.

METASTATIC OSTEOSARCOMA

Approximately 20% of patients with osteosarcoma present with clinically overt metastatic disease, and the presence of metastatic disease is clearly a strong predictor of poor outcome.[5] Gene expression profiling through the use of complementary DNA microarray analysis is beginning to uncover the molecular events that dictate metastatic potential as well as poor response to chemotherapy,[102,103] findings that may pave the way for future molecularly targeted therapies.

Ezrin

Using complementary DNA microarrays to identify metastasis-related genes that are differentially expressed between murine cell lines with differential metastatic potential, Khanna et al[103] identified the membrane-cytoskeleton linker ezrin, a member of the ERM (ezrin/radixin/moesin) group of proteins that is expressed in a variety of cancers, some of which are associated with poor outcome.[104,105] Ezrin is believed to be involved in intracellular signal transduction involving cell migration and metastasis.[106] In a study of 19 patients with osteosarcoma, Khanna et al[107] subsequently showed that the disease-free interval of pediatric patients with high ezrin immunohistochemical staining was significantly shorter than that in patients with low ezrin staining, and that the risk of metastatic relapse was 80% greater in the former group.

Annexin 2

Gillette et al[108] used a molecular subtraction technique called representational difference analysis to identify genes differentially expressed between primary human osteosarcomas and subsequent metastatic lesions. Among the genes found to be down-regulated in metastatic samples was annexin 2 (*anxA2*).[108] Annexin 2 belongs to a large family of diverse proteins that are characterized by conserved annexin repeat domains and the ability to bind negatively charged phospholipids in a calcium-dependent manner.[109] Annexin 2 is thought to play a role in cancer and metastases, but studies to date have not been clear regarding whether this role is inhibitory or positive.[102,110] They also showed that *anxA2* was down-regulated in a subset of human osteosarcoma metastases and metastatic cell lines at both the RNA and the protein level of expression. Although overexpression of *anxA2* did not alter cell motility, adhesion, or proliferation of osteoblastic cells, they did observe an increase in differentiation. This led them to propose that perhaps through disruption of the differentiation program, perhaps through loss of *anxA2*, a proliferative advantage could be conferred to promote the aggressiveness of osteosarcoma tumors.

CXCR4/SDF-1

The chemokine stromal cell-derived factor 1 (SDF-1) belongs to a family of cytokine-like proteins that play a role in cytoskeleton rearrangement, adhesion to endothelial cells, and directional migration. It is expressed on the surface of vascular endothelial cells and is secreted by various tissues, such as bone marrow, lung, and liver.[111] Its chemotactic effect is mediated by interaction with the chemokine receptor 4 (CXCR4). The CXCR4/SDF-1 axis has been shown to be important in tumor progression for certain tumors, including breast, prostate, lymphoma, and rhabdomyosarcoma.[112,113] To investigate whether this system could play a role in osteosarcoma metastasis and homing to the lungs, Perissinotto et al[114] analyzed the effects of SDF-1 on migration, adhesion, and proliferation of osteosarcoma cells and showed that migration and adhesion were promoted by SDF-1 treatment. They also showed in a mouse system that development of pulmonary metastasis after injection of osteosarcoma cells could be prevented by the administration of T134 peptide, an inhibitor of CXCR4.

Fas Signaling Pathway

Fas ligand is a transmembrane protein that induces apoptosis in susceptible cells by interacting with its receptor, Fas, a member of the tumor necrosis factor

receptor family. Developing resistance to this pathway is one mechanism that is commonly exploited by tumors to acquire metastatic potential.[115,116] The Fas/Fas ligand receptor–mediated cell death pathway has been implicated in the metastatic behavior of osteosarcomas.[117] In one study using an experimental nude mouse model, a metastatic osteosarcoma cell line with low Fas expression was injected into the mice, and re-expressing Fas in these cells caused a significantly lower number of metastatic lung tumor nodules than those occurring in control cell lines.[118] Increasing Fas expression increased the sensitivity of those osteosarcoma cells to Fas-induced cell death. Thus, loss of Fas expression may be one mechanism by which osteosarcoma cells may evade host resistance mechanisms in the lung, where they most often metastasize, and suggests that this pathway serves as a potential therapeutic target for osteosarcoma.

DISCUSSION

Unlike other pediatric sarcomas characterized by specific molecular defects that can aid in both diagnosis and prognosis, osteosarcoma is characterized by a complex array of cytogenetic abnormalities as well as by alterations in multiple different molecular pathways. Few predictive measures exist at the time of diagnosis to help determine which patients will have a better clinical outcome in response to therapy. Currently, the best measures of outcome are the absence of overt metastatic disease at diagnosis and the response to neoadjuvant chemotherapy.[4,5] Therefore, understanding the molecular mechanisms underlying the pathogenesis of osteosarcoma and the crucial factors determining metastatic potential will aid in discovering better clinical predictors of outcome and in devising more rational and targeted therapies for this disease.

Many different pathways are now known to be involved in osteosarcoma biology, including those dealing with cell cycle regulation, maintenance of genomic integrity, and transcriptional regulation, and it is likely that multiple pathways are contributing to the pathogenesis of osteosarcoma, either in concert or in parallel. There is growing evidence that different members of the various pathways mentioned earlier, including p53, RECQ helicase, and telomere maintenance pathways, interact physically and functionally with each other. For example, it is known that p53 interacts with the RECQ helicases. WRN is bound by p53 at its carboxy terminus,[119] and WRN potentiates p53-mediated transcription from p53-responsive promoters.[120] p53-mediated apoptosis is also attenuated in fibroblasts from patients with Werner syndrome when compared with control fibroblasts, suggesting a role of WRN in this process as well.[119] In turn, p53 modulates both the helicase activity and the exonuclease activity of the WRN protein. It inhibits the unwinding of synthetic Holliday junctions by WRN[121] and also inhibits the exonuclease activity of WRN in a dose-dependent manner.[122] Thus, this physical interaction between p53 and WRN is capable of influencing the activities of both partners. p53 also interacts with the BLM protein physically and functionally. Bloom syndrome cells have attenuated p53-dependent apoptosis similar to that in WRN cells, and BLM and p53 appear to cooperate in regulating apoptosis,[123] as well as in regulating transcription and cell growth control.[124] p53 can inhibit the helicase function of BLM, and current data suggest that p53 may regulate homologous recombination through its modulation of interactions between BLM and recombination intermediates.[125,126] Recently, it has been shown that p53 regulates the transcription of *RECQL4* by repressing its promoter activity.[127] In addition to the interaction of p53 with RECQ helicases, members within the RECQ helicase family itself are also known to interact with each other,[128,129] and it is likely that the individual members function in concert in some roles (e.g., in homologous recombination pathways) and independently in other roles, which may help explain the basis of the clinical differences, including tumor spectra, observed between patients with the different RECQ helicase syndromes.

Members of the RECQ helicase family have recently also been shown to interact with proteins in the telomere maintenance pathways that are also implicated in osteosarcoma biology. For example, the telomere maintenance protein TRF2 binds both WRN and BLM and stimulates the helicases activities of these proteins.[130] In the presence of replication protein A, WRN and BLM can actively unwind long telomeric duplex regions that are bound by TRF2. WRN is also known to interact via its *N*-terminus with the Ku heterodimer,[131] which locates to telomeric ends,[132] where it plays a role in telomere maintenance in addition to its many other roles. The Ku complex can stimulate the exonuclease activity of WRN,[133] an interaction that may be biologically important. The BLM protein also interacts with both TRF1 and TRF2, and its helicase activity is regulated in vitro by these telomeric proteins.[134] BLM may also play a role in the ALT pathway of telomere maintenance, which is frequently utilized by osteosarcomas. BLM co-localizes with telomeric foci in ALT cells and affects telomeric DNA synthesis through its helicase activity.[135] These data, along with that of ongoing studies, suggest coordination between the activities in the RECQ helicase and telomere maintenance pathways, both of which are likely to play a role in the pathogenesis of osteosarcoma.

The challenges that lie ahead for osteosarcoma biology will be to understand exactly how the different pathways mentioned herein interact functionally and how they intersect with each other in timing during tumor formation and progression. Bona fide tumor suppressor genes such as *Rb* and *p53* are considered to be part of a subgroup of cancer susceptibility genes ("gatekeepers")[136] whose inactivation represents a threshold event in tumorigenesis.[37] Other tumor suppressors (e.g., the RECQ helicases and telomere maintenance proteins) function more as "caretakers" whose inactivation leads to multiple genetic alterations due to loss of genomic integrity. As mentioned earlier,[37] *RB* and *p53* also have caretaker functions in the maintenance of genomic stability and as such play multiple roles in limiting the process of tumorigenesis. Perhaps there are critical initiating events, such as mutations in the p53 or RB genes, that initiate tumorigenesis, and other subsequent events, such as alterations in the RECQ or telomere pathways, that occur later to help establish tumor formation. Once established, other factors, including oncogene overexpression and increased signaling through metastatic pathways, can then play a role in the progression and spread of tumors[35] (Fig. 1). Dissecting the various interactions between pathways involved in cell cycle regulation, DNA metabolism, and maintenance of genomic integrity and understanding the role of oncogenes and metastatic pathways, as well as drug resistance pathways, will eventually provide a rational strategy for devising specific therapies that target the pathways leading to osteosarcoma.

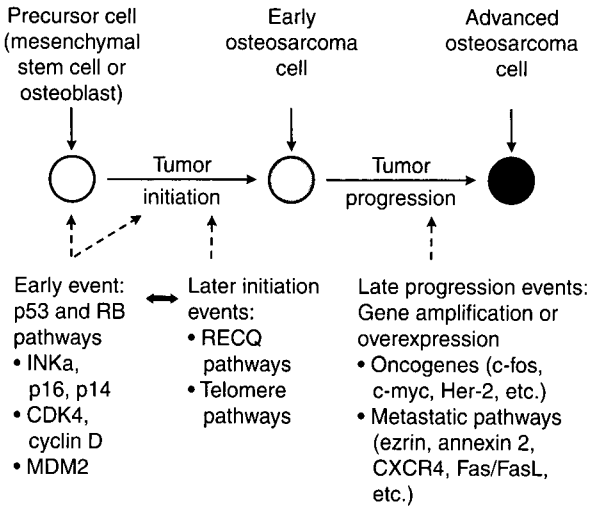

FIGURE 1 Schematic working model of somatic events leading to the molecular pathogenesis of osteosarcoma. Examples of some of the potential pathways that may be altered during this process are listed in the text. The exact timing of these events is unknown (dotted arrows), as are the specific interactions (↔) between different pathways. Most osteosarcomas are diagnosed at a late stage and therefore have already accumulated complex molecular cytogenetic alterations.

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