Critical Signaling Pathways in Bone Sarcoma: Candidates for Therapeutic Interventions

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Bone sarcomas cause disproportionate morbidity and mortality and desperately need new therapies as there has been little improvement in outcomes in 20 years. Identification of critical signaling pathways, including type 1 insulin-like growth factor receptor (IGF-1R) for Ewing sarcoma and possibly osteosarcoma, and the ERBB and the Wnt signaling pathways for osteosarcoma, have emerged as receptors mediating vital signals for bone sarcoma. Akt, mammalian target of rapamycin (mTOR), phosphoinositide 3-kinases, mitogen-activated protein kinase kinase, extracellular signal-regulated kinases, and Ras pathway play key roles in at least some tumors, and inhibition of mTOR in particular will likely lead to improved survival, although clinical trials are still underway. The Notch pathway and ezrin are essential for osteosarcoma metastasis, and Fas down-regulation is necessary for survival of metastases in lungs. As little is known about chondrosarcoma signaling, more preclinical work is needed. By defining vital signaling pathways in bone sarcomas, small molecule inhibitors can be applied rationally, leading to longer survival and reducing morbidity and late effects from intensive chemotherapy.

Introduction

Bone sarcomas arise in about 2500 Americans every year, consisting primarily of three histologic types: osteosarcoma, Ewing sarcoma, and chondrosarcoma. Osteosarcoma and Ewing sarcoma have a peak incidence during the adolescent growth spurt, whereas chondrosarcoma occurs more often in adults. All three bone sarcomas are of mesenchymal origin, but may have different "normal" precursors or counterparts: the osteoblast for osteosarcoma and the chondrocyte for chondrosarcoma. The progenitor cell for Ewing sarcoma is not clearly established, but may be the mesenchymal stem cell itself. A role for chemotherapy has been clearly established for osteosarcoma and Ewing sarcoma, in which intensive cytotoxic treatment is necessary to prevent future recurrence. This need reflects the presence of established micrometastatic disease in most patients in whom metastasis is not yet clinically evident. Chondrosarcoma remains primarily a surgical disease, although nearly half of patients may show some chemotherapy responsiveness. Although chemotherapy agents and surgical approaches vary by disease and anatomic location, all three bone sarcomas share a common feature: the survival curves for these diseases have remained disturbingly flat for the past two decades and, despite numerous clinical trials and diverse agents tried, it appears unlikely that further benefit will be achieved by changes in systemic chemotherapy. For this reason, it is imperative to explore novel targeted therapies for bone sarcomas, basing our treatment approaches on better knowledge of the biology of each disease. Understanding signaling pathways in each bone sarcoma may aid in personalizing therapeutics to increase patient survival.

Pathways

Receptor tyrosine kinases

Insulin-like growth factor (IGF)-1 and IGF-2 bind to IGF-1 receptor (IGF-1R). This receptor has two α and two β subunits that are connected through disulfide bonds; the receptor also can heterodimerize with insulin receptor (IR). After dimerization and ligand binding, the receptors undergo adenosine triphosphate–dependent phosphorylation at specific residues that form docking sites for effector molecules such as IR substrate 1 (IRS-1) to bind IGF-1R or IR. This binding, which also may result in phosphorylation of the second messenger protein, results in activation of a signaling cascade. IRS-1 activates phosphoinositide 3-kinases (PI3K), which causes activation of Akt. Akt mediates activation of multiple survival pathways (Fig. 1). IGF-1 signaling

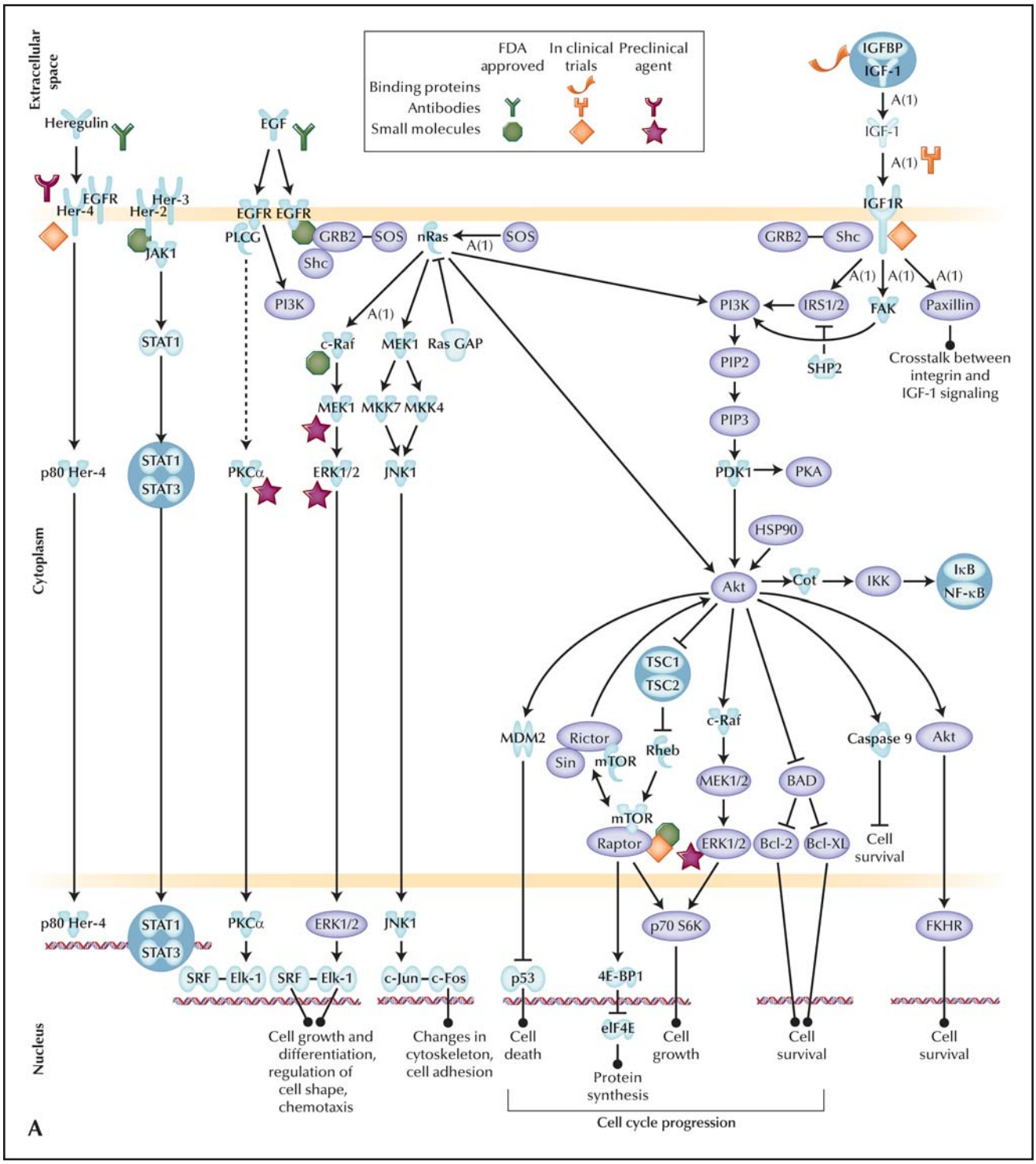

Figure 1. Key signaling pathways in bone sarcoma and selected inhibitors. **A** and **B**, In general, signaling commences with binding of a ligand to a receptor tyrosine kinase (eg, epidermal growth factor receptor [EGFR] or other ERBB family member, type 1 insulin-like growth factor receptor [IGF-1R]) or other cell-surface receptor. Activation of the receptor tyrosine kinase initiates activation of multiple downstream signaling pathways, usually several different pathways from a particular receptor. The downstream pathways activated by receptor tyrosine kinases are similar despite different receptors, and several key nodes—including Ras, phosphoinositide 3-kinases (PI3K), Akt, and Src—are common to most pathways, though not all of these secondary pathways may be important in a particular cell line. The signaling from vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and other receptor tyrosine kinases is similar to that shown for IGF-1R and EGFR here. Potential areas of crosstalk among different receptors are shown. Signaling may be inhibited by binding proteins that remove ligand (*crescents*), antibodies to receptors or to ligands (*T or Y structures*), or by small molecule inhibitors of the specific enzyme (*stars*). Some common inhibitors are shown. *Octagons* indicate US Food and Drug Administration (FDA)–approved drugs; *diamonds* indicate agents in clinical trials; and *stars* indicate preclinical agents. 4E-BP1—4E binding protein 1; BAD—Bcl2-antagonist of cell death; CK—casein kinase; CoA—coenzyme A;

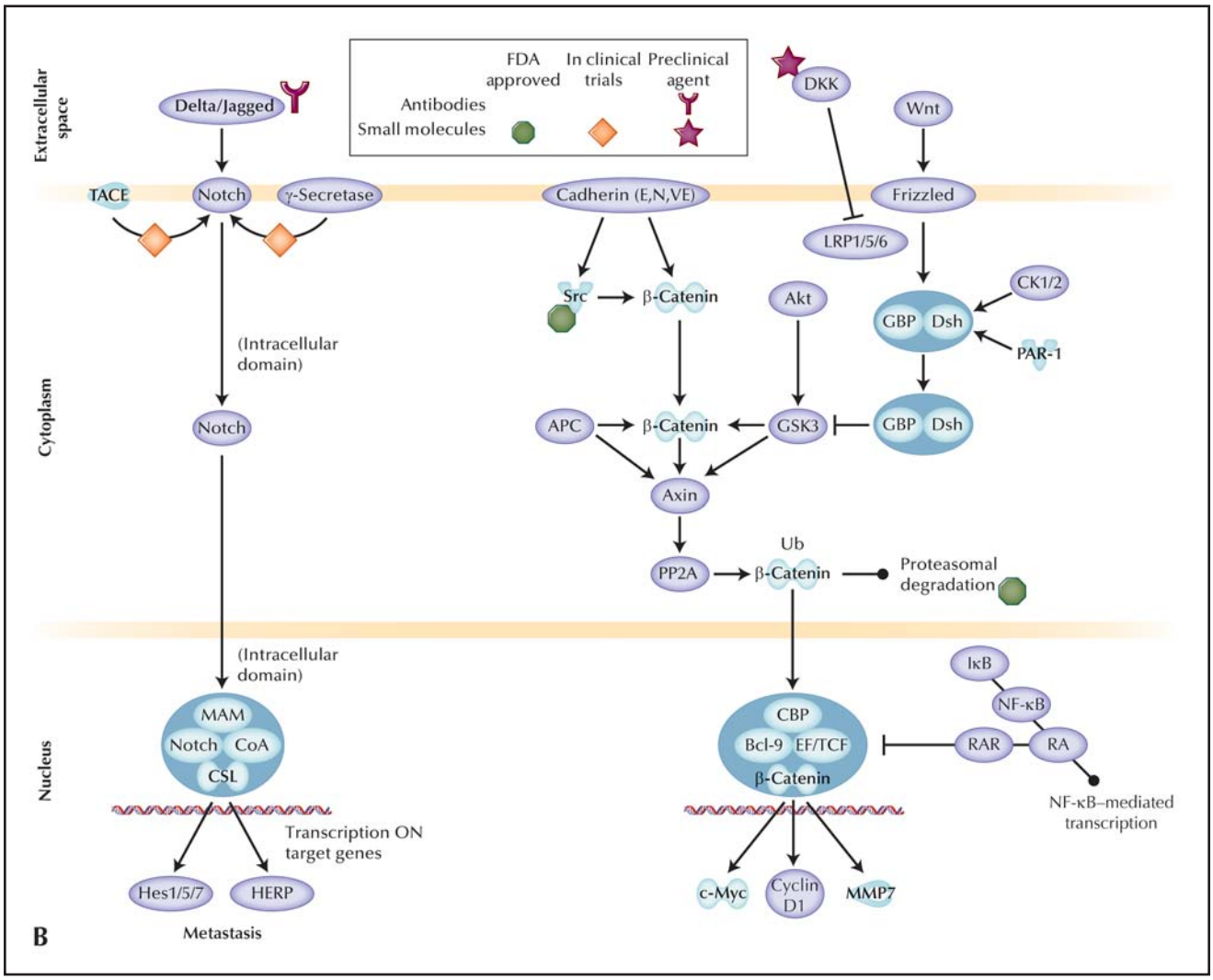

Figure 1. ***(Continued)*** E—epithelial; EF/TCF—enhancer factor/T-cell factor ; eIF4E—eukaryotic initiation factor 4E; ERK1/2—extracellular signal-regulated kinases 1 and 2; FAK—focal adhesion kinase; FKHR—forkhead box O1; GBP—glycogen synthase kinase 3 binding protein; GRB2—growth factor receptor-bound protein 2; GSK3—glycogen synthase kinase 3; Her—human epidermal growth factor receptor; IGFBP—insulin-like growth factor binding protein; IKK—IκB kinase; IRS—insulin receptor substrate; JNK—Jun kinase; MEK—mitogen-activated protein kinase kinase; MEKK—MEK kinase; mTOR—mammalian target of rapamycin; N—neural; NF-κB—nuclear factor κ-light-chain-enhancer of activated B cells; p—chromosome; P—placental; PIP—phosphatidyl inositol phosphate; PP2A—protein phosphatase 2A; PKCα—protein kinase C α; RAR—retinoic acid receptor; Rheb—Ras homolog enriched in brain; SOS—son of sevenless; SRF—serum response factor; STAT—signal transducer and activator of transcription; VE—vascular endothelial.

can also activate the Ras extracellular signal-regulated kinase (ERK)1/2 pathway by recruitment of the Shc protein after phosphorylation of specific tyrosine residues. Kim et al. [1••] reviewed IGF-1R in pediatric cancers.

Growth factors bind to ERBB protein family receptors, which stabilizes ERBB homo- or heterodimers and induces autophosphorylation [2]. There are four receptors in this family: epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (Her-2), Her-3, and Her-4. Her-2 has no known ligands, but is the preferred pairing partner for the other ERBB receptors, whereas Her-3 does not have a transactivation domain but can provide the strongest signaling in heterodimers. These receptors may recruit effector molecules, such as growth factor receptor-bound protein 2 (GRB2) and Src, depending on which tyrosine residues are phosphorylated. GRB2 activates son of sevenless (SOS), which activate membrane-bound Ras. Ezrin may assist with Ras activation, especially in osteosarcoma [3]. Ras can activate multiple signals, including Raf-1 and PI3K. Activation of Raf-1 results in phosphorylation and activation of mitogen-activated protein kinase kinase 1/2 (MEK1/2), which then activates ERK. ERK translocates into the nucleus in which it activates transcription factors such as myc and c-fos. Src activation can lead to further increases in Ras and PI3K signaling, as well as signals related to motility and cell shape through regulation of focal adhesion kinase. Signaling of other receptor tyrosine kinases (RTKs), including the platelet-derived growth factor receptors, fibroblast growth factor receptor, c-Met, the various colony-stimulating factor receptors, and the vascular endothelial growth factor receptor (VEGFR), is similar to ERBB signaling. There is

crosstalk and overlap between the signaling cascades that normally leads to increased growth and survival signals.

mTOR

The mammalian target of rapamycin (mTOR) pathway is activated downstream of multiple RTK pathways such as platelet-derived growth factor (PDGF), ERBB, IGF-1R, particularly through Akt. Rheb allows for activation of mTOR-Raptor (mTORC1) signaling, and is inhibited by *TSC2*. *TSC2* is inhibited by Akt and ERK and activated by glycogen synthase kinase 3 (GSK3). Activation of mTOR-Raptor signaling results in activation of hypoxia-inducible factor 1, which promotes angiogenesis, and 4E binding protein 1 (4EBP-1) and p70S6K, which result in increased cellular metabolism and growth. The mTORC1 complex is inhibited directly by rapamycin and its analogs. In the mTORC2 complex, mTOR is associated with rapamycin-insensitive companion of mTOR, and may cause activation of Akt, Rho, and protein kinase C (PKC) α. The mTORC2 complex is not inhibited directly by rapamycin, and treatment with rapamycin may cause an increase in mTOR-mediated Akt activity, presumably through increased Sin. Thus rapamycin may cause an unexpected increase in proliferation in some tumors caused by unopposed mTORC2. Inhibition strategies for mTOR have been reviewed recently [4].

Notch

There are four notch receptors in humans: Notch 1 to 4 [5•]. The Notch ligands are grouped into two families, the Jagged and Delta-like ligands, binding of which triggers two proteolytic cleavage of Notch, resulting in release of the intracellular domain. The first proteolytic cleavage is mediated by the ADAM10 protease on the extracellular side of Notch receptor and the second by γ-secretase, cleaving within the transmembrane domain. After being cleaved by γ-secretase, the intracellular domain enters the nucleus to affect gene expression. In normal organogenesis and embryogenesis, Notch plays diverse roles and is crucial for dorsal–ventral patterning, limb bud formation, and control of proliferation and apoptosis in processes such as digital ray formation. In malignancy, Notch signaling can function as an oncogene or tumor suppressor, depending on the malignant tissue type. For cancers driven by Notch activity (ie, T-cell leukemias and lymphomas), clinical trials are underway using γ-secretase inhibitors (GSI) to block Notch pathway activation. The results of these studies will need careful interpretation, however, because GSI inhibitors affect many pathways other than Notch, and proteins arising from translocations of the Notch locus with other genes may not inhibited by GSI.

Wnt

Wnt proteins bind Frizzled family receptors, activating the Dishevelled family of proteins (Dvl). When Wnt is absent, β-catenin is phosphorylated by GSK3, facilitating β-catenin ubiquitination and destruction. In the presence of Wnt, *LRP* binds Fizzled (Fz), Wnt, and Dvl into an active complex that inhibits GSK3, thereby blocking destruction of β-catenin and increasing β-catenin signaling. Phosphorylation of β-catenin by GSK3 leads to the destruction of β-catenin. *DKK* inhibits *LRP* and therefore decreases β-catenin signaling in the nucleus. Many components of this pathway are mediated by or mediate other signaling cascades. GSK3 is inhibited by AKT. GSK3 also activates *TSC2*, which inhibits mTOR-Raptor signaling, thereby reducing cancer cell metabolism and growth. As Wnt signaling is critical in normal bone development [6], it is not surprising that this pathway has important functions in bone sarcomas.

Death receptors

A key development in the understanding of programmed cell death was the identification of specific cell-surface proteins whose signals could initiate apoptosis. These receptors, members of the tumor necrosis factor (TNF) receptor family, include Fas (CD99) and TNF-related apoptosis-inducing ligand receptor (TRAIL-R). The Fas ligand trimer (FASL), which is abundant in lung, binds Fas receptors and results in trimerization of the receptor. Fas-associated death domain (FADD) binds to the death domain of Fas, creating the death-inducing signaling complex. This facilitates binding of caspase-8, resulting in its activation. Caspase-8 can then activate the intrinsic and extrinsic apoptotic pathways to result in apoptosis. Signaling from TRAIL-R is similar [7].

Pathways summary

Many signaling molecules are overexpressed, mutated, constitutively activated, or in some cases downregulated to result in cancer. There is overlap and crosstalk in the signaling of these molecules, which explains why it may be necessary to block more than one signaling cascade to achieve therapeutic benefit in children with sarcoma.

Osteosarcoma

Osteosarcoma is the most common primary bone cancer, with a peak incidence during the adolescent growth spurt. It is characterized by the presence of malignant osteoid, usually together with spindle cells, and accounts for more than 10% of malignancies of adolescents [8]. The disease is characterized by severe chromosomal instability, aneuploidy, and heterogeneity within the tumor. Because osteosarcomas have a high mutation frequency, we expect that treatment of these tumors with novel selective small molecule inhibitors as monotherapy will result in resistance mutations in a high proportion of patients. p53 and retinoblastoma (Rb) tumor suppressor pathways are often involved in osteosarcoma pathogenesis [9]. Most tumors demonstrate combined inactivation of p53 and Rb, and osteosarcoma often develops in individuals with congenital defects of either of these genes

[10]. *MDM2* amplification and p53 mutation may reflect tumor progression, but no correlation between alteration and response to chemotherapy or patient survival has been demonstrated [11].

Osteosarcoma cells express functional IGF-1R on their cell surface, which stimulates the cells to proliferate [12]. In vitro, IGF-1–dependent growth can be inhibited using monoclonal antibodies or antisense oligonucleotides against IFG-1R [12]. This was further tested in vivo, using a humanized anti–IGF-1R antibody, which resulted in tumor regression in two osteosarcoma xenograft models [13]. Clinical trials using anti–IGF-1R antibodies for patients with osteosarcoma are in early phase trials, and combination trials are forthcoming.

Most osteosarcoma samples express both EGFR and EGF, facilitating constitutive EGFR activation [14,15]. This activation may have important functional consequences, because in one study an EGFR inhibitor reduced motility, colony formation, and invasiveness in five osteosarcoma cell lines tested, whereas inhibitors of Her-2, nerve growth factor receptor (NGF-R), and PDGF receptor (PDGF-R) had no effect on these properties [16]. Alterations in c-fos have been found to occur more frequently in osteosarcoma patients with recurrent or metastatic disease, probably because of direct RTK signaling and crosstalk among other signaling pathways that amplify c-fos. These patients may be candidates for more aggressive combinational therapies. Src inhibitors such as dasatinib, which appeared to reduce in vitro motility and in vitro surrogate measures of metastasis, such as matrigel invasion, did not reduce metastasis in vivo [17••]. The failure of dasatinib to reduce in vivo metastasis is likely related to redundancy of pathways downstream from Src, and underscores the need for thorough preclinical laboratory studies on novel therapies for bone cancers before pursuing widespread clinical trials with these agents. These observations further suggest that multiple pathways may require simultaneous inhibition to decrease osteosarcoma metastasis.

Nitrogen-containing bisphosphonates are antiresorptive agents with secondary activities on cancer cells. They inhibit growth through downregulation of protein prenylation. Prenylation is the addition of a hydrophobic tail to the C-terminus of a protein to increase interaction with membranes and other proteins. There are two forms of prenylation, farnesylation and geranylgeranylation. Direct antitumor activity of nitrogen-containing bisphosphonates correlates with their efficacy against osteoclast-mediated bone resorption, which correlates with their inhibition of farnesyl diphosphate synthesis [18]. These agents induce apoptosis by activating caspase-3-like proteases, bypassing p53 and Rb mutations [19], and significantly reducing cell invasion in osteosarcoma cells in a dose-dependent manner [20•].

Ras is a lipid-anchored small GTPase that must be prenylated to associate with membranes and become activated. Ras can mediate oncogenic signals in many cancer types and frequently has oncogenic mutations in carcinomas, although oncogenic Ras has never been observed in osteosarcoma. Ras signaling can result in cell growth and proliferation or cell death signals depending on the duration and cellular context [21•]. Bisphosphonates inhibit this process efficiently only at the tumor–bone interface, and may be useful in preventing new bone metastasis. A clinical trial is underway within the Children's Oncology Group assessing the feasibility of combining bisphosphonate therapy with conventional chemotherapy for newly diagnosed osteosarcoma patients presenting with metastatic disease. At the Children's Cancer Hospital at M. D. Anderson, we have treated many recurrent osteosarcoma patients with zoledronic acid together with a variety of chemotherapy and have not observed increased toxicities. Interestingly, these patients rarely develop new bone metastases once bisphosphonate treatment is begun. Inhibition of farnesylation by the small molecule inhibitor tipifarnib resulted in increased Ras activation and decreased cell survival, possibly through activation of ERK or p38 [22]. Cisplatin and chelerythrine also have been identified as inducing osteosarcoma cell death through Ras-mediated activation of ERK [21,23].

mTOR signaling plays a key role in cell metabolism. Blocking mTOR signaling may reduce growth and metastasis in osteosarcoma patients by blocking S6 kinase 1 and 4EBP-1 phosphorylation [24]. The mTOR inhibitors rapamycin and its derivatives are powerful antitumor compounds. Rapamycin has been found to inhibit ezrin-mediated metastatic behavior, likely caused by the direct interaction of ezrin with PI3K [25,26]. Blocking mTOR activity also causes growth arrest of cells in G1 phase of cell cycle [20], and was reported to induce partial response of some patients who received the rapamycin analog AP23573 in phase 2 trial [27]. In the Children's Cancer Hospital at M. D. Anderson, we have used rapamycin successfully together with liposomal doxorubicin and bevacizumab for refractory osteosarcoma patients, and have seen metabolic remissions by positron emission tomography scan that have been durable for many months. Rapamycin also appears to help render osteosarcoma metastases more radiosensitive in our experience.

Hes1 signaling, induced by Notch pathway activation, is a key regulator of metastatic potential in osteosarcoma [28••]. Using either GSI or molecular approaches, Hes-1 downregulation caused reduced invasion in vitro and reduced metastasis in vivo. Other researchers subsequently made similar observations [29,30]. Because Notch signaling is so critical for osteoblast function, it is not surprising that it is also important for osteosarcoma. Therapies targeting the Notch pathway or genes induced by it may prove beneficial therapeutically. In addition, ezrin expression is required for osteosarcoma metastasis. Although there is no clear link between ezrin and the Notch pathway defined at present, ezrin has been linked to PKC [3].

The Wnt pathway may contribute to the metastatic potential of osteosarcoma. Components of Wnt signaling control

both osteoblast and osteoclast differentiation, playing a significant role in bone development and homeostasis [31]. Expression of various Wnt pathway members may have prognostic importance, and evidence suggests that Wnt pathway signaling induces metastasis in osteosarcoma. In one series of 44 osteosarcoma patients from Memorial Sloan-Kettering, half of the patients expressed the Wnt coreceptor *LRP5*, and these patients had a much higher rate of metastasis than did the *LRP5*-negative patients [32]. This is presumably the same patient series in which Her-2 expression was associated with metastasis, though there is no analysis about coexpression of Her-2 and *LRP5*, and no analysis of variance was done to assess independent predictive value. In a similar analysis by the group at Georgetown, also examining 44 patient samples, Wnt10b was observed in 75%, with a trend toward decreased survival in these patients [33]. Another study found elevated levels of the Wnt inhibitor *DKK1* in the serum of pediatric osteosarcoma patients and at the edges of tumors, and that this expression suppressed the normal osteoblasts, preventing repair of tumor-induced bone lysis [34].

Low levels of Fas expression in osteosarcoma lung metastases correlate with poor prognosis [35••]. Immunohistochemistry from lung nodules of osteosarcoma patients indicates that all but one of 38 samples tested were Fas negative or weakly Fas positive [36••]. Chemotherapeutic agents, such as gemcitabine, can upregulate Fas expression in the lung and induce regression of lung metastasis in vivo [37].

Methylation of gene promoter regions is a mechanism for inactivation of tumor suppressor genes. Aberrant methylation or epigenetic silencing is a mechanism for the loss of function of several tumor suppressor genes. Hou et al. [38] compared the profile of 30 pairs (normal and osteosarcoma) of tissue samples tested for methylation differences of several genes. They found hypermethylation of *RASSF1A*, *MGMT*, *GSTP1*, *APC*, *DAPK1*, *CDH1* in the osteosarcoma tissue samples. Furthermore, the degree of hypermethylation was associated with patient outcome. *RASSF1A* associates with Ras to induce apoptosis. An additional study found *RASSF1A* was not expressed in 40% primary osteosarcoma patient samples and in 83.3% of osteosarcoma cell lines tested. This suggests that inactivation of *RASSF1A* is frequent event in osteosarcoma and may have an important role in tumorigenesis [39]. Treatment with DNA methylation inhibitor reactivated transcription of *RASSF1A* in the *RASSF1A*-negative cell lines. Inactivation of *RASSF1A* may be an alternate mechanism to shift Ras activities toward growth in the absence of Ras mutations. These data support interest in further preclinical evaluation of methylation inhibitors in osteosarcoma.

Ewing Sarcoma

Ewing sarcoma is the second most common bone cancer, with a worse overall prognosis than osteosarcoma. Ewing sarcoma is characterized by chromosomal translocation involving the *EWS* gene on chromosome 22q12 and a member of the ets transcription factor family, most often with the *FLI1* gene on chromosome 11q24 (85% of patients), leading to aberrant *EWS*–ets transcription factor [40].

The most exciting recent development in Ewing sarcoma biology and therapy has been the development of agents targeting IGF-1R. Expression of IGF-1R is high in Ewing sarcoma, and *FLI1* oncoprotein requires IGF-1R to transform murine fibroblasts [41]. The small molecule inhibitor IGF-1R kinase inhibitor NVP-AEW541 delayed tumor formation in xenograft mice [42]. The anti–IGF-1R antibody SCH 717454 caused tumor regression in a Ewing sarcoma xenograft model [13]. Some patients showed sustained clinical remission in phase 1 trials using IGF-1R antibodies [43]. However, the results from IGF-1R inhibition in preclinical models and clinical trials tend not to be durable—that is, resistant disease eventually develops from monotherapy. This resistance likely arises from other pathways being recruited to compensate for IGF-1R inhibition [1••]. Therefore, inhibition of IGF-1R may need to be combined with inhibition of other signaling pathways important for progression of this disease.

The ERBB family may also play an important role in Ewing sarcoma and, surprisingly, Her-4 may be the most important family member for Ewing sarcoma. Her-4 activates PI3K–AKT pathway and this can be associated with increased resistance of cells to cytotoxic agents. In one study, Ewing sarcoma cell lines were grown under contact-free conditions, under which they formed microspheroids that may more closely resemble normal growth conditions in vivo. This culture method resulted in increased chemotherapy resistance that was dependent upon both E-cadherin expression and Her-4 activation of PI3K [44•]. Inhibition of E-cadherin adhesion or downregulating Her-4 or its downstream PI3K pathway may provide a means of reducing Ewing sarcoma cell growth, survival, and metastasis.

One potential targeted therapy for Ewing Sarcoma is to inhibit the tumor blood supply by blocking VEGF. VEGF drives new blood vessel formation in Ewing sarcoma, because there is a positive correlation between VEGF and microvessel density (MVD) [45]. Blockade of VEGF production with an siRNA inhibited the growth of Ewing sarcoma xenografts, and production of VEGF in Ewing sarcoma also recruits $CD34^+$ cells, precursors to pericytes, to the growing vasculature [46,47]. Anti-VEGF also inhibited the growth of Ewing sarcoma xenografts [47]. Unfortunately, VEGF-mediated vascularization is not the only signal promoting a blood supply in Ewing sarcoma because stromal cell-derived factor-1α (SDF-1α)–enhanced tumor neovascularization and growth in the absence of VEGF expression [46]. Therefore, SDF-1 signaling may be an alternate pathway that could mediate resistance to anti-VEGF therapy by Ewing sarcoma following therapy.

Other potential therapeutic targets in Ewing sarcoma include Wnt pathway signaling [48] and the Src family

kinase Lyn [49]. As the biology of Ewing sarcoma becomes better defined, further targets are likely to emerge. It is expected that most of these novel therapies will require use in combination with more traditional cytotoxic treatments to have their best effect.

Chondrosarcoma

Chondrosarcomas have a better prognosis than osteosarcoma but are very resistant to chemotherapy [8]. Molecular prognostic markers have not been identified thus histologic grading is the best prognostic indicator of disease progression [50]. No genetic alterations in c-myc, N-myc, or c-fos have been identified [8]. Most data generated on chondrosarcoma have been restricted to immunohistochemistry. Larger studies with additional methods beyond immunohistochemistry are needed to confirm the small amount of data about this disease and to bring the hope of novel small molecule medicines to this rare but important disease [50].

Conclusions

Traditional systemic cytotoxic therapy for bone sarcomas likely has provided all the improvement in clinical outcome we are likely to see from these agents. Different combinations or higher doses are unlikely to improve patient survival. To reduce both the mortality and morbidity associated with these diseases, we need a better understanding of the biology underpinning each tumor type. The past several years have shown great strides in understanding both osteosarcoma and Ewing sarcoma, and the benefit of our increased knowledge is now reaching the clinic. The coming years should bring even more significant changes, provided that resources continue to support necessary research.

Disclosure

No potential conflicts of interest relevant to this article were reported.

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