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Cytogenetics and Molecular Biology of Osteosarcoma

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Osteosarcoma is a relatively uncommon malignancy with about 1000 new cases diagnosed each year in the United States. It is, however, among the most common nonhematologic primary malignant tumors of bone in both children and adults (Bell and Siegal, 2002). The peak incidence of osteosarcoma occurs in the second decade with an additional smaller peak after age 50 (Dorfman and Czerniak, 1995). The tumors typically arise in the metaphyseal regions of long bones, with the distal femur, proximal tibia, and proximal humerus representing the three most common sites (Dahlin and Coventry, 1967; Marcove et al, 1970).

Many types of osteosarcoma are currently recognized (Table 1), with classification primarily based on location of the lesion, associated bone (for example, the gnathic bones), or related disease entity. The conventional type, arising in the intramedullary cavity of the bone, represents approximately 75% of all osteosarcomas (Mertens et al, 1993). These tumors frequently penetrate and destroy the cortex of the bone and extend into the surrounding soft tissues. The typical gross appearance of these lesions is variable, with fragments of bone admixed with softer tissues that have a chondroid to fibrous consistency. Areas of necrosis are common, especially in specimens obtained after preoperative chemotherapy. The histologic appearance of conventional osteosarcoma allows this group to be further subdivided based on the primary differentiation of the mesenchymal component present. The most commonly recognized subtypes are osteoblastic, chondroblastic, and fibroblastic, with a number of less commonly observed patterns including epithelioid, giant-cell rich, small cell, and telangiectatic. The unifying histologic feature present in all types and subtypes of osteosarcoma is the presence of tumor osteoid produced by the neoplastic cells.

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The diagnosis of osteosarcoma requires a combination of clinical presentation, radiologic studies, and pathologic tissue evaluation. Although serum alkaline phosphatase may be elevated, laboratory studies are generally not helpful in establishing the diagnosis. The initial clinical symptom of these tumors is frequently pain in the affected area, which may also be associated with localized soft tissue swelling or limitation of motion in the adjacent joint (Dahlin and Coventry, 1967). Radiologic evaluation is vital in making the correct diagnosis. Typical findings include a lytic intramedullary lesion with scattered areas of new bone formation, often with destruction of the bony cortex and extension of mass into the surrounding soft tissue (Kumar et al, 1987; Lindbom et al, 1961). Reactive new bone formation may be seen under the periosteum forming a "Codman angle," or "Codman's triangle," and invasion into adjacent soft tissues may produce a "sunburst" pattern of periosteal reaction (Ragsdale et al, 1981). Initial pathologic assessment is frequently performed on biopsy material in which typical histologic features together with the appropriate radiologic findings allow for the definitive diagnosis.

Case Presentation

The patient was an 18-year-old white woman in excellent health except for a history of asthma who presented initially with left-sided shoulder pain that radiated to her fingers. The pain had been present for approximately 1 year, was aggravated by activity, and was not associated with a known injury. Radiographs revealed an aggressive lytic lesion in the proximal left humerus with permeative destruction of the humeral shaft and foci of mineralized lesional content (Fig. 1). A computerized tomography also showed the permeative process involving the proximal humeral diaphysis and the anatomic neck with an area of intralesional increased density suggestive of poorly organized osteoid (Fig. 2). A magnetic resonance imaging scan showed the extent of marrow replacement by the neoplasm, with increased T2-weighted intramedullary signal and enhancement with intravenous contrast (Fig. 3, A and B). She subsequently underwent an open biopsy that demonstrated an atypical cartilagi-

Table 1. Types of Osteosarcoma

Conventional (intramedullary) osteosarcoma
Osteoblastic
Chondroblastic
Fibroblastic
Epithelioid
Giant-cell rich
Small cell
Telangiectatic
Cortex-associated osteosarcoma
Parosteal
Dedifferentiated parosteal
Periosteal
High-grade surface
Intracortical
Low-grade (central) osteosarcomas
Osteoblastoma-like osteosarcomas
Disease-associated osteosarcoma
Osteosarcoma in Paget's disease
Osteosarcoma in fibrous dysplasia
Osteosarcomas in Mazabraud's disease
Multicentric osteosarcomas
Post-irradiation osteosarcoma
Osteosarcoma of the gnathic bones

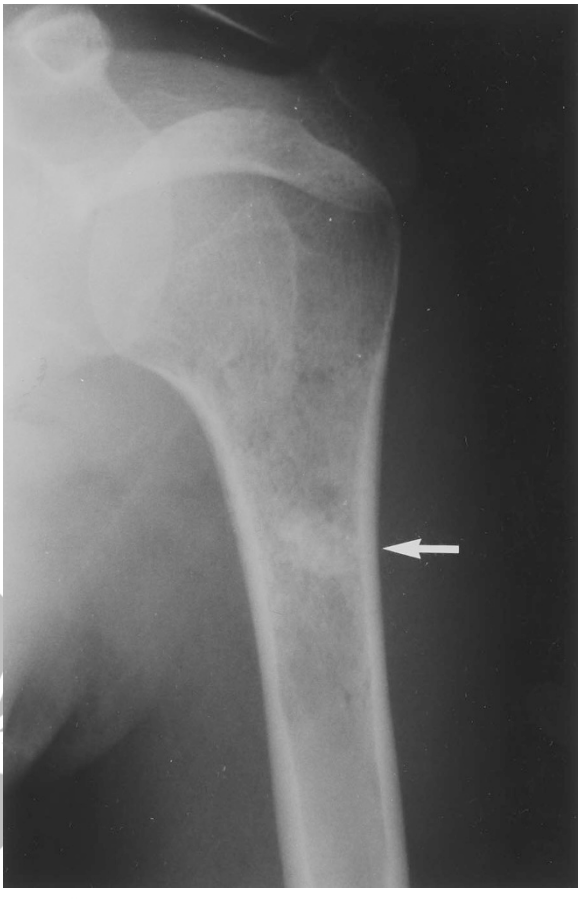

Figure 1.
Anterior-Posterior (AP) radiograph of the left proximal humerus. Note the area of ill-defined sclerosis (*arrow*) within a predominantly permeative lytic lesion of the proximal shaft.

nous proliferation with hypercellularity and pleomorphism of cellular constituents. The large chondroblasts were seen to have nuclei with "open chromatin" and increased binucleate forms, and increased mitotic figures were easily identified (Fig. 4). No tumor osteoid was identified on the biopsy material, but because of the patient demographics and radiologic appearance, osteosarcoma was suspected, specifically, chondroblastic osteosarcoma.

The patient underwent definitive surgical resection of the tumor followed by postoperative chemotherapy. Gross examination of the resected specimen revealed a 9.1-cm intramedullary mass with a tan fibro-osseous to glistening cartilaginous cut surface (Fig. 5). The tumor was centered in the metadiaphyseal portion of the proximal humerus with focal extension into the periosteal soft tissue. Microscopically, two dominant patterns of neoplastic cells were identified. One consisted of a small fibroblast-like spindle cell population with significant pleomorphism, high mitotic rate (>5 per high-power field), karyorrhexis, and tumor osteoid production with occasional tumor giant cells and osteoclastic giant cells (Fig. 6A). The second component was composed of a malignant cartilaginous component similar in appearance to the biopsy specimen (Fig. 6B). Tumor osteoid was identified between the lobules of cartilage. The diagnosis of a mixed fibroblastic and chondroblastic (conventional) osteosarcoma was made, and all margins of resection were seen to be free of tumor.

Molecular Biology

Numerous cytogenetic and molecular studies of osteosarcoma have been undertaken in recent years that have yielded varied and often conflicting results. Thus far, these studies have been of limited value in diagnosing, prognosticating, and understanding the molecular events driving tumorigenesis in osteosarcoma. Molecular studies of this tumor face the technical challenges of obtaining adequate material after preoperative necrotizing chemotherapy and the need for decalcification of specimens. Additionally, the overall rarity of these tumors contributes significantly to the difficulty in studying them. Nonetheless, much progress has been made in this field over the past two decades, which is contributing to our knowledge of the etiology of osteosarcoma and continues to yield important information suggesting potential targets for prognostic biomarkers and for novel gene therapy.

Cytogenetics and DNA Analysis

It was evident from the earliest studies of ploidy (DNA content) in osteosarcomas that these tumors showed a significant propensity toward aneuploidy (Bauer et al, 1988; Hiddemann et al, 1987; Kreicbergs et al, 1984; Kruzelock et al, 1997). This is particularly true of high-grade lesions, in which 92 (96%) of 96 high-grade tumors were seen to be hyperploid, whereas all 4 of the low-grade, parosteal osteosarco-

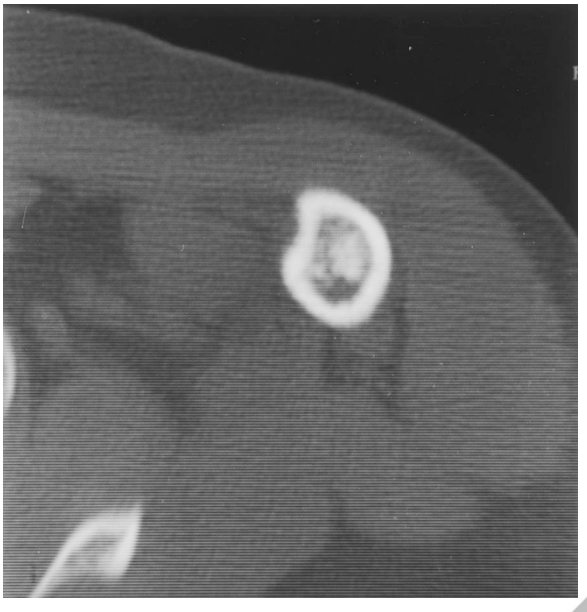

Figure 2.
Computerized axial tomographic image of the proximal left humerus. There is an area of intramedullary increased yet ill-defined sclerosis consistent with a focus of mineralized osteoid.

mas studied were found to be diploid (Bauer et al, 1988). Others have examined the association between the degree of aneuploidy and prognosis and response to chemotherapy (Bosing et al, 1987; Kusuzaki et al, 1999; Look et al, 1988). One report demonstrated that, among hyperploid tumors, the presence of near-diploid stem lines was associated with a better prognosis, including both a lower incidence of pulmonary metastasis and an improved disease-free survival after treatment (Look et al, 1988). Another showed a higher frequency of aneuploidy after preoperative chemotherapy among tumors exhibiting a poor response (Bosing et al, 1987). However, contrary to these earlier reports, a more recent study reported that patients whose tumors showed a nondiploid DNA content had a longer event-free survival after surgical resection and chemotherapy than did those with diploid tumors, suggesting a better response to chemotherapy in the former (Kusuzaki et al, 1999). Thus, the exact role of aneuploid DNA content in relation to prognosis and response to therapy remains unsettled.

Conventional cytogenetic analysis of osteosarcomas has yielded an enormous number and variety of karyotypic alterations. Boehm et al (2000) recently examined the cytogenetic profile of 36 cases and reviewed many previously published ones. They found chromosomal abnormalities in 25 (69%) of 36 cases, ranging from near-diploid to near-tetraploid tumors as well as many specimens that showed multiple clones with different degrees of ploidy. This frequency and complexity of alterations is similar to that previously shown by Bridge et al (1997), who reported 47 (64%) of 73 cases to have chromosomal anomalies ranging from haploid to near-hexaploid DNA content. Other reports also confirm these findings (Fletcher et al, 1994; Mertens et al, 1993). Of 161 cases examined by Boehm et al (2000), the most commonly identified numeric chromosomal abnormalities were gain of chromosome 1 and loss of chromosomes 9, 10, 13, and 17. The most common chromosomal structural rearrangements (found in 20 or more of the 161 cases) included 1p11-13, 1q11-12, 1q21-22, 11p14-15, 14p11-13, 15p11-13, 17p, and 19q13. Also identified were a high percentage of chromosomes that could not be readily assigned, which, the authors note, limit the ability of conventional cytogenetics to completely assess the various aberrations found in osteosarcoma.

In more recent years, comparative genomic hybridization (CGH) has contributed additional insights to conventional cytogenetic techniques in examining chromosomal anomalies in osteosarcoma. DNA sequence copy number increases have been identified in association with 1q21, 3q26, 6p, 8q, 12q12-13, 14q24-qter, 17p11-12, Xp11.2-21, and Xq12 (Forus et al, 1995; Hulsebos et al, 1997; Tarkkanen et al, 1995, 1999). Additionally, DNA sequence loss of 2q, 6q, 8p, and 10p have been commonly identified (Tarkkanen et al, 1995). Of these chromosomal abnormalities, copy number increases at 8q, 1q, and 17p seem to be of particular interest. Patients with copy number increases at 8q (specifically 8q21.3-22 and 8cen-q13) demonstrate both a poorer disease-free survival and a shorter overall survival, whereas patients with a copy number increase at 1q21 showed a trend toward a shorter overall survival (Tarkkanen et al, 1999). The 17p amplicon identified by CGH is also a common structural abnormality identified by conventional cytogenetic techniques. Interestingly, despite its overall amplification, segments of DNA loss have been identified within the 17p amplicon (Hulsebos et al, 1997; Wolf et al, 1999), indicating that loss of genetic material and not just gene amplification may be an important factor for development of osteosarcoma within this amplicon.

A few subtypes of osteosarcoma have shown a more limited or specific set of cytogenetic changes that may provide some insight into their development in particular as well as the development of osteosarcoma in general. Although areas of gene amplification, such as ring chromosomes, double minutes, and homogeneously staining regions, are fairly common in conventional osteosarcomas, the presence of ring chromosomes is frequently the sole cytogenetic alteration or one of only a few abnormalities found in parosteal osteosarcomas, one of the few recognized low-grade lesions associated with osteosarcomas (Bridge et al, 1997; Fletcher et al, 1994; Sinovic et al, 1992). CGH has revealed that these ring chromosomes in parosteal osteosarcoma are associated with amplification of DNA at chromosome 12q13-15 (Szymanska et al, 1996), a region harboring CDK4, MDM2, SAS, and other potential oncogenes (Gamberi et al, 2000; Ragazzini et al, 1999). Cytogenetic and CGH analyses of a small number of low-grade central osteosarcomas have demonstrated similar gain of chromosomal material at 12q13-14 as well as abnormalities involving chromosome 6p (Bridge et al, 1997; Grubb et al, 1999; Tarkkanen et al, 1998). Finally, translocation of chromosomes 11 and 22, identical to that seen in Ewing's sarcoma, has been demonstrated in

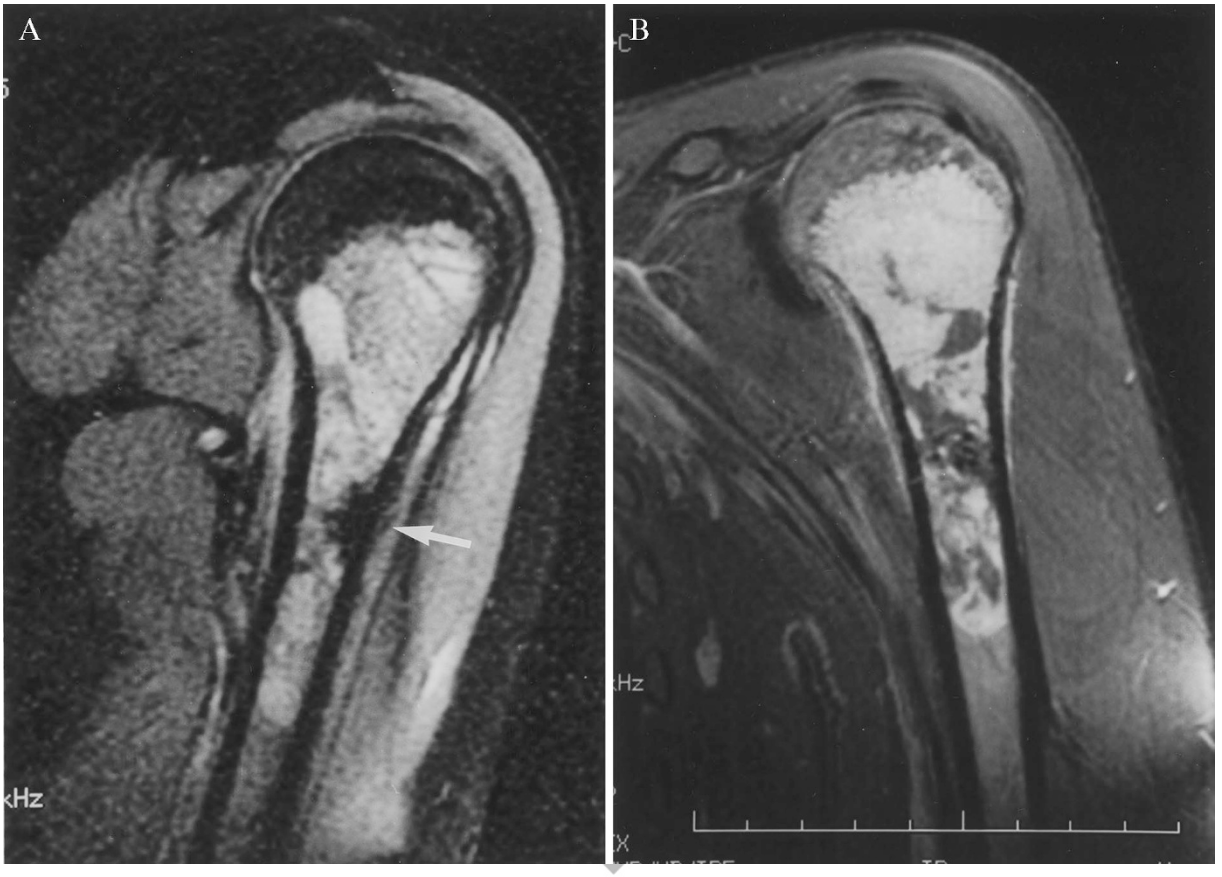

Figure 3.
A, Coronal T2-weighted fat-suppressed magnetic resonance image of the proximal left humerus. The intramedullary process has increased T2-weighted signal. Note absence of signal from foci of mineralized osteoid (*arrow*). B, Coronal T1-weighted fat-suppressed image magnetic resonance image of the proximal left humerus after intravenous contrast administration shows marked contrast enhancement.

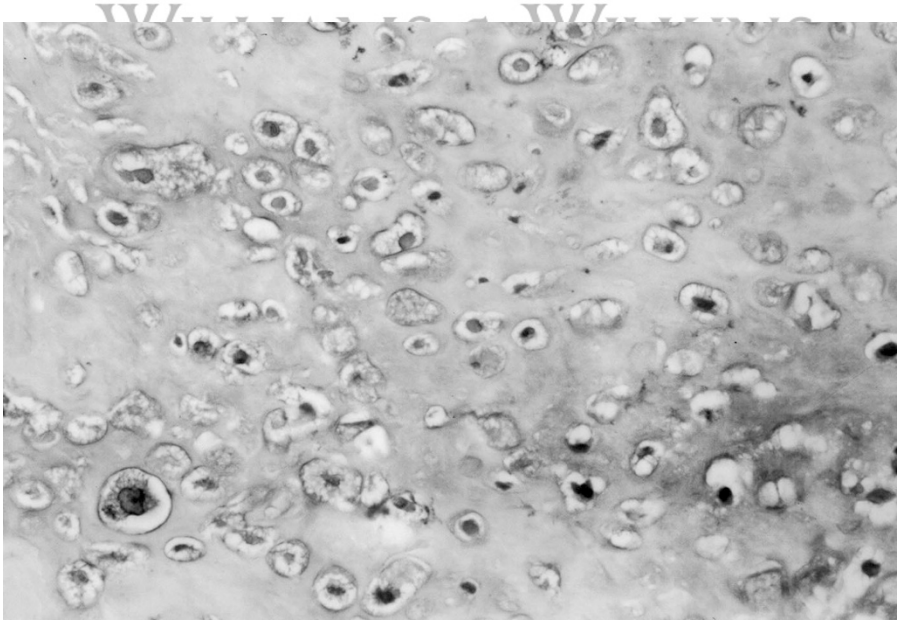

Figure 4.
High-power photomicrograph of seemingly "pure" cartilaginous neoplasm.

small cell osteosarcoma (Noguera et al, 1990) and accompanies immunophenotypic and ultrastructural similarities between these two entities (Bell and Siegal, 2002).

Gene Studies

The search for specific genes associated with the development of osteosarcoma has stemmed from

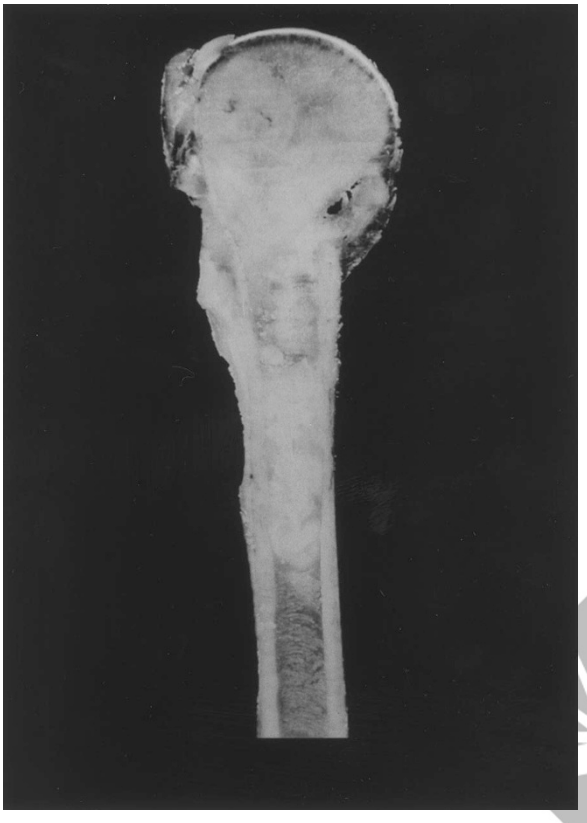

Figure 5.
Bivalved humerus resection specimen. Although metaphyseal centered, the neoplasm is seen to fill the entire bone from the subchondral epiphysis to the diaphyseal shaft. Note the cortical destruction with elevation of the periosteum in the metaphyseal region (Codman's triangle).

both prior cytogenetic findings as well as diseases associated with osteosarcoma. Although not necessarily specific to osteosarcoma, a number of important genes have been identified, including various tumor suppressors genes, oncogenes, and genes coding for growth factors. Many of the abnormalities identified have also been detected in other sarcomas as well as some carcinomas. The relative importance of many of these factors to the tumorigenesis of osteosarcoma remains unresolved, even as the relationship of certain gene expression profiles to prognosis and response to chemotherapeutics becomes more evident.

Perhaps the best characterized of the signal transduction pathway defects found in osteosarcoma are those associated with the retinoblastoma (RB) gene. An association between osteosarcoma and RB is well recognized, with patients affected by hereditary RB having up to 1000 times the incidence of osteosarcoma compared with the general population (Abramson et al, 1984; Kitchin and Ellsworth, 1974). Additionally, sporadic osteosarcomas show alterations of the RB gene in approximately 70% of cases (Ladanyi and Gorlick, 2000). Loss of heterozygosity (LOH) at the RB gene locus on chromosome 13 is present in about 60% to 70% of tumors (Belchis et al, 1996; Feugeas et al, 1996; Yamaguchi et al, 1992), whereas structural rearrangements and point mutations occur less commonly (Araki et al, 1991; Miller et al, 1996; Wunder et al, 1991). Lastly, LOH at the RB locus has been proposed as a poor prognostic factor in osteosarcoma (Benassi et al, 1999; Feugeas et al, 1996).

The RB gene functions as a tumor suppressor by acting as the major regulator of the G1 to S phase progression in the cell cycle. The RB protein accomplishes this by binding to and thus suppressing the function of the E2F transcription factor. The ability of the RB protein to bind E2F is controlled by phosphorylation, which is mediated primarily by D-type cyclin-dependent kinases, in particular the cyclin D1/CDK4 complex. The p16 protein product of the INK4A gene in turn inhibits CDK4. Thus, RB and p16 function to suppress cell proliferation while cyclin D1 and CDK4 act to promote proliferation. (Ladanyi and Gorlick, 2000; Nevins, 2001). Defects in each of these genes of the RB pathway are thought to have a potential role in the development of osteosarcoma (Ladanyi and Gorlick, 2000).

As discussed above, abnormalities of the RB gene are commonly seen in osteosarcoma, however, anomalies of p16, CDK4, and cyclin D1 are also well documented. INK4A gene deletions and loss of p16 expression are found in approximately 10% to 15% of tumors (Belchis et al, 2000; Maitra et al, 2001; Nielsen et al, 1998), and loss of p16 expression has been shown to correlate with decreased survival in pediatric osteosarcomas (Maitra et al, 2001). Amplification of the q13-15 region of chromosome 12, which contains the CDK4 gene as well as other potential oncogenes including MDM2 and SAS, has been identified in about 10% of osteosarcomas (Tarkkanen et al, 1995). Likewise, CDK4 gene amplification has been demonstrated by Southern blot analysis in a similar percentage (9%) of cases (Maelandsmo et al, 1995; Wei et al, 1999). Additionally, amplification of cyclin D1 has been found in a smaller number of cases (Maelandsmo et al, 1995; Wei et al, 1999).

Another tumor suppressor gene thought to be important in the development of osteosarcoma is the p53 gene. The p53 gene is located on chromosome 17p13 (McBride et al, 1986), an area frequently identified as abnormal in osteosarcomas by cytogenetics and CGH, as discussed above. The p53 gene product is a transcription factor that induces the transcription of many genes that are involved in cell cycle control as well as apoptosis (Hung and Anderson, 1997). Alterations of the p53 gene can occur through allelic loss, gene rearrangements, or point mutations. Point mutations of the p53 gene are predominantly missense mutations, whose products can presumably form heterodimers with, and inactivate, normal p53 molecules (Ladanyi and Gorlick, 2000). Many reports have identified abnormalities of the p53 gene in osteosarcoma, with frequencies of up to 50% of cases (Guo et al, 1996; Lonardo et al, 1997; Masuda et al, 1987; Miller et al, 1996; Sztan et al, 1997). Further evidence of the association of p53 with osteosarcoma is provided by the high risk of bone sarcomas in patients with the Li-Fraumeni syndrome who have a germline mutation of p53 (Li et al, 1988; Srivastava et al, 1990). Additionally, germline mutations of p53 have been identified in

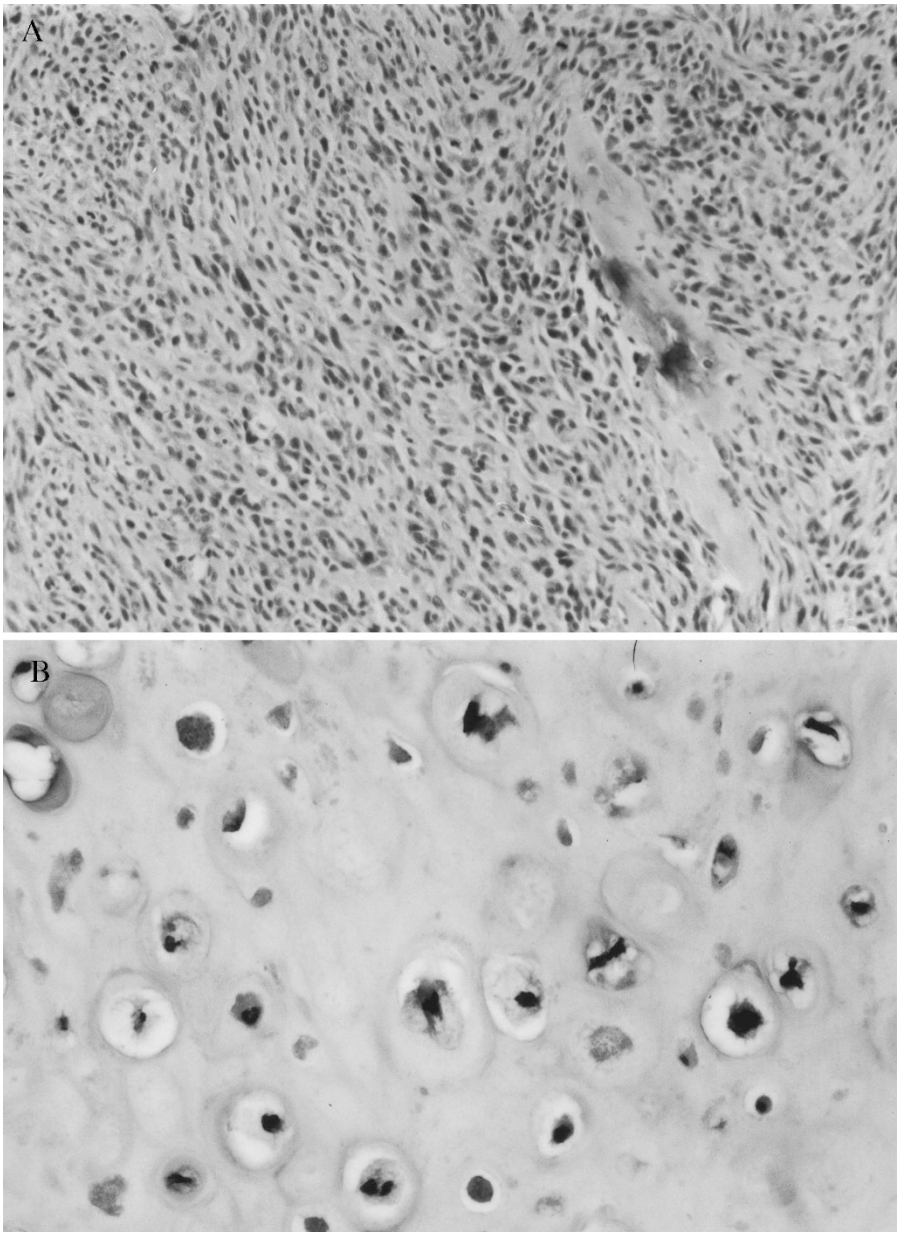

Figure 6.

A, Photomicrograph of definitive specimen with spindled fibrosarcomatous-like tumor. Note the partially mineralized osteoid on the right side, off center. B, Photomicrograph of the malignant cartilaginous component from the same definitive specimen. The histologic appearance mimics that of the original biopsy (Fig. 4).

a small percentage of patients with sporadic osteosarcoma (Toguchida et al, 1992). Alterations at the p53 locus may have prognostic significance because these changes may indicate a decreased sensitivity to chemotherapeutic agents (Goto et al, 1998).

As with the RB gene, other genes are involved in the regulation of p53, and abnormalities of these have similarly been identified in cases of osteosarcoma. The MDM2 gene, located on chromosome 12q13 along with CDK4, encodes a protein that binds p53 and blocks the activity of the latter (Ladanyi and Gorlick, 2000). Overexpression of MDM2 in osteosarcomas has been identified and may provide an alternative mechanism for disruption of the normal p53 pathway (Lonardo et al, 1997; Miller et al, 1996). Another potentially important protein involved in this pathway is the p14 product of the INK4A gene, which is the same gene producing the p16 protein involved in the RB pathway. The p14 protein exerts a protective effect on p53 by binding to the MDM2 gene product (Ladanyi and Gorlick, 2000). Thus, INK4A deletions affecting p16, as discussed above, would also affect p14 and lead to both decreased p53 and RB activity.

Tumor suppressor genes other than p53 and RB are suspected to be associated with osteosarcoma. High frequencies of allelic loss have been show at 3q, 13q, 17p, and 18q, suggesting that, in addition to p53 (17p) and RB (13q), two other tumor suppressor genes may exist at 3q and 18q (Yamaguchi et al, 1992). Interestingly, expression of the DCC (deleted in colon cancer)

gene, which is located on chromosome 18q21, has been shown to be decreased in a high percentage of both high- and low-grade tumors (Horstmann et al, 1997). In addition, abnormalities of chromosome 18q are implicated in a familial predisposition to osteitis deformans (Paget's disease), a bone condition leading to a several thousand-fold increased risk of developing osteosarcoma (Wick et al, 1981), with both sporadic and Pagetic tumors demonstrating LOH at 18q (Hansen et al, 1999). A possible tumor suppressor gene involved in osteosarcoma tumorigenesis has also been localized to a region of chromosome 3q26 (Kruzelock et al, 1997).

A number of other oncogenes are known to be abnormally expressed in cases of osteosarcoma. The SAS gene, located on chromosome 12q13 along with CDK4 and MDM2, was reported to be amplified in 36% of osteosarcomas and 100% of parosteal osteosarcomas (Noble-Topham et al, 1996). SAS encodes a transmembrane protein believed to be involved in cellular growth processes (Ladanyi and Gorlick, 2000). The proto-oncogene c-fos has been shown to be expressed at high levels in up to 61% of osteosarcomas (Wu et al, 1990). Additionally, c-fos overexpression is seen more often in tumors from patients in whom metastases develop (42%) as opposed to those from patients who remain disease free (17%) (Gamberi et al, 1998). Similarly, c-myc is overexpressed in patients in whom metastatic disease develops (42%), more so than in patients who remain free of disease (23%) (Gamberi et al, 1998). HER2/neu (c-erbB-2) expression has been observed in just over 40% of cases and has been associated with early pulmonary metastases and decreased survival (Gorlick et al, 1999; Onda et al, 1996). Many other growth factor–related proteins have been shown to be overexpressed in cases of osteosarcoma including TGFβ-1, TGFβ-3, IGF-1, and the Met/HGF receptor-ligand pair (Burrow et al, 1998; Ferracini et al, 1995; Franchi et al, 1998; Kloen et al, 1997). The exact importance of these remains to be elucidated, and the possibility that these merely represent a generalized "dys-regulation" associated with neoplastic transformation cannot be completely excluded.

Resistance of osteosarcomas to chemotherapeutics commonly used to treat the disease has also been linked to specific genetic alterations. High-dose methotrexate resistance is believed to be linked to decreased drug uptake by either a defect in the reduced folate carrier or to alterations or amplifications of the drug target, dihydrofolate reductase (Guo et al, 1999). Decreased expression of the reduced folate carrier has been found in 65% of specimens at initial biopsy, and this decreased expression was identified in a significantly higher percent of tumors showing a poor response to chemotherapy (Guo et al, 1999). Increased dihydrofolate reductase expression was found in only a small percent (10%) of specimens at initial biopsy, however, increased expression was demonstrated in 62% of cases at the time of definitive surgery or relapse, suggesting a possible mechanism of acquired methotrexate resistance (Guo et al, 1999). The ATP-dependent transmembrane protein, p-glycoprotein, has been associated with chemoresistance to a number of drugs including doxorubicin, an important drug in the treatment of osteosarcomas (Baldini et al, 1999). Overexpression of p-glycoprotein is present in approximately 40% of tumors and has been shown to relate to a poorer prognosis (Baldini et al, 1999; Chan et al, 1997).

Conclusions

Although no specific diagnostic cytogenetic or molecular marker has yet been identified for osteosarcoma, much knowledge has been gained in this area over the past decade. Many important cytogenetic findings related to important chromosomes and regions of chromosomes have led to the identification of genes associated with the disease. While the exact molecular pathogenesis remains unclear, alterations of genes and gene pathways such as RB and p53 have been well established as contributing to the genesis of osteosarcoma; and many other genes also seem to be involved. Important strides are just now beginning to be made in identifying abnormalities that have prognostic and therapeutic implications that may be useful in guiding patient therapy.

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