CDKN2A Gene Deletions and Loss of p16 Expression Occur in Osteosarcomas That Lack RB Alterations

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Osteosarcomas often suffer mutations of the *RB* (retinoblastoma) gene, with resultant inactivation of the pRb protein. pRb is one component in a cell-cycle control pathway that includes the p16 (encoded by the *CDKN2A* gene) and cyclin-dependent kinase 4 (cdk4, encoded by the *CDK4* gene) proteins. We therefore sought to determine whether the *CDKN2A* and *CDK4* genes were altered in those osteosarcomas that lacked *RB* inactivation. Twenty-one osteosarcomas (2 low-grade and 19 high-grade) were evaluated for homozygous deletion of the *CDKN2A* gene, *CDK4* amplification, and allelic loss of the *RB* gene, as well as for expression of p16 and pRb proteins. Five high-grade osteosarcomas showed loss of p16 expression; four of these had homozygous *CDKN2A* deletions, and the fifth had a probable deletion obscured by numerous nonneoplastic, p16-immunopositive multinucleated giant cells. Thus, p16 immunohistochemistry may provide a sensitive means for assessing *CDKN2A* status. Twelve tumors (including the two low-grade osteosarcomas) were immunopositive for pRb, and nine tumors were immunonegative for pRb. Of the five cases with *CDKN2A*/p16 alterations, none had allelic loss of the *RB* gene and all expressed pRb, suggesting that each of these tumors had an intact *RB* gene. None of the tumors showed *CDK4* amplification. No alterations were detected in the two low-grade osteosarcomas. This study suggests that *CDKN2A* is a tumor suppressor inactivated in osteosarcomas that lack *RB* mutations and that the p16-pRb cell-cycle control pathway is deregulated in a large number of high-grade osteosarcomas. (*Am J Pathol* 1998, 153:159–163)

ring chromosomes in parosteal osteosarcomas,¹ karyotypic changes in osteosarcomas are often complex and include a variety of structural and numerical abnormalities with frequent losses of chromosomes 13q, 17p, 18q, and 3q.^{2–9} Of the known tumor suppressor genes at these loci, the *RB* gene on chromosome 13q has been most clearly implicated in osteosarcoma tumorigenesis. *RB* mutations occur in a large number of sporadic osteosarcomas and patients with germline *RB* mutations have an increased incidence of osteosarcomas.^{10–15}

The pRb protein encoded by the *RB* gene functions as part of a cell cycle regulatory pathway that also involves the p16 protein (encoded by the *CDKN2A* gene on chromosome 9p21) and cyclin-dependent kinase 4 (cdk4, encoded by the *CDK4* gene on chromosome 12q13). p16 appears to inhibit the function of cdk4-cyclin D1 complexes, which regulate pRb through phosphorylation. Phosphorylation inactivates *RB*, thereby allowing entry of the cell into S phase with resultant cellular proliferation. Thus, either inactivation of p16 or overexpression of cdk4 could promote tumorigenesis in a manner similar to that of pRb inactivation. Accordingly, the various components of this pathway are deregulated in a large number of human cancers.^{16–23}

Although *RB* mutations are a well-recognized oncogenic event in osteosarcomas, the role of the *CDKN2A* and *CDK4* genes in osteosarcoma formation have not been clearly defined, particularly in relation to *RB*/pRb abnormalities. We therefore evaluated a series of osteosarcomas for alterations of these key cell cycle regulatory molecules.

Materials and Methods

Materials

Twenty-one tumors were frozen at the time of biopsy (19 patients) or primary resection (2 patients). No patient had received preoperative chemotherapy or radiation therapy. The patient population consisted of 12 males and 9 females who ranged in age from 9 to 77 (average 26) years old. Two tumors were low-grade (parosteal) osteo-

Genetic changes underlying the initiation and progression of osteosarcomas are poorly understood. Except for

Accepted for publication April 2, 1998.

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sarcomas that arose from the posterior surface of the distal femur. Nineteen tumors were high-grade (12 osteoblastic, 3 mixed osteoblastic/chondroblastic, 2 giant cell rich, 1 chondroblastic, and 1 telangiectatic). One of the giant cell-rich osteosarcomas arose in a patient with known Paget's disease of bone. The tumors were located in the tibia (6), femur (5), pelvis (4), fibula (2), and humerus (2). DNA was extracted from frozen tumor tissues according to standard phenol-chloroform procedures. Before DNA extraction, all tumor tissues were examined by frozen section to ensure that they contained viable tumor tissue.

Homozygous Deletions of CDKN2A

To assay for homozygous deletions of the *CDKN2A* gene, we used a comparative multiplex polymerase chain reaction technique.²³ The products were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light by ethidium bromide staining. This assay has been titrated to detect homozygous *CDKN2A* deletions in tumors with less than 30% contaminating nonneoplastic cells.²³

CDK4 Gene Amplification

CDK4 gene amplification was evaluated using a differential polymerase chain reaction assay.^{23,24} The products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Positive controls included glioblastomas with known *CDK4* amplification.²³

Allelic Loss of the RB Gene

Allelic loss of chromosome 13q14 at the *RB* gene was assessed by analysis of the *RB* 1.20 polymorphism in intron 20 of the *RB* gene as detailed elsewhere.²⁵ Because only tumor tissue was available, tumors with two *RB* alleles could be scored as maintaining both alleles, but cases with one allele were scored as indeterminate, either representing allelic loss or being noninformative.

p16 Immunohistochemistry

The JC8 anti-p16 mouse monoclonal IgG2a antibody was generated in the Massachusetts General Hospital Cancer Center and recognizes an epitope in the first ankyrin repeat (amino acids 1–32) of the p16 protein. The antibody detects a single 16-kd band on Western blots of human tissues, including brain tumors (J. Koh, unpublished data), and has been used in the immunohistochemical evaluation of human brain tumors.²⁶ Formalin-fixed, paraffin-embedded tissues were sectioned at 6 μ m onto Probe-On Plus slides. After baking at 65°C for 1 hour, the sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersing the slides in 0.5% hydrogen peroxide in methanol for 5 minutes between the

100% and 90% alcohol steps. An antigen retrieval step was used, consisting of microwaving the slides in 0.01 mol/L sodium citrate (pH 6.0) for three changes of 5 minutes each, followed by cooling in phosphate-buffered saline (PBS) rinses. The sections were incubated in 10% normal horse serum in 5% milk for 20 minutes at room temperature. The JC8 anti-p16 antibody was applied at a 1:500 dilution in 1% bovine serum albumin/PBS and incubated in a humidity chamber at room temperature for 2 hours. After the primary antibody incubation, a secondary biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) was applied at a 1:1000 dilution (in 1% bovine serum albumin/PBS) for 1 hour at room temperature, followed by the avidin-biotin complex kit (ABC Elite, Vector Laboratories) also for 1 hour at room temperature. Between each of the preceding three steps, slides were washed in three changes of PBS. After the application of 0.06% diaminobenzidine (Sigma Chemical Co., St. Louis, MO) with 0.01% H₂O₂ for 3 minutes, the slides were washed in distilled water and lightly counterstained in the hematoxylin solution Gill No. 1 (Sigma). After dehydration in graded alcohols and clearing in xylene, the slides were coverslipped. Tonsil tissue served as a control in which nuclear and cytoplasmic staining was noted specifically in histiocytic cells in germinal centers and epithelial cells of the mucosal lining. Negative controls were performed by omitting the primary antibody and by using an irrelevant mouse monoclonal antibody.

pRb Immunohistochemistry

The pRb immunohistochemical protocol was similar to the above p16 assay, with minor variations. Blocking of endogenous peroxidase activity in H₂O₂/methanol was carried out for 30 minutes. The slides were initially incubated with 10% normal horse serum in 1% bovine serum albumin/PBS for 30 minutes. The primary mouse monoclonal anti-pRb antibody (G3-245, Pharmingen) was diluted 1:2500 and applied overnight at 4°C. The secondary biotinylated horse anti-mouse antibody was diluted at 1:1000 and applied for 1 hour at room temperature. In tonsils, there was distinct nuclear immunohistochemical expression of pRb in germinal centers and in basal epithelial layers.

Tissue for p16 and pRb immunohistochemical staining was available from the original biopsy or resection specimen in 18 cases; in three cases, the original biopsy slides contained insufficient material for immunohistochemistry, in which cases it was performed on the resection specimen after the patients had received preoperative chemotherapy.

Results

CDKN2A Genetic Analysis and p16 Immunohistochemistry

Homozygous deletion of *CDKN2A* was detected in 4 of 21 osteosarcomas (Figure 1). All 4 tumors with deletions were high-grade; no deletion was detected in the 2 low-

Figure 1. Comparative multiplex analysis of the *CDKN2A* gene. Homozygous deletion of the gene is present in four tumors (lanes 4–7, top), as evidenced by preferential amplification of the control amplicon (bottom band) with minimal amplification of the *CDKN2A* amplicon (top band); all these tumors were immunonegative for p16. The lower panel (lanes 3 and 4) shows no deletion in two osteosarcomas that were immunopositive for p16. **Lanes 1:** pUC18/*Hae*III digest size marker; **Lanes 2:** negative controls ("no DNA" lanes); **Lane 3, top:** normal control DNA.

grade (parosteal) osteosarcomas. All tumors with *CDKN2A* deletions were immunonegative for p16 protein expression (Figure 2b). The tumor cells in one additional giant cell-rich osteosarcoma were immunohistochemically negative for p16. In that tumor, however, numerous benign giant cells were immunopositive for p16, suggesting that the apparent lack of *CDKN2A* deletion may have been a false negative polymerase chain reaction result (see Discussion) (Figure 2e). The remaining 16 tumors lacked *CDKN2A* homozygous deletion and were immunopositive for p16 (Figure 2a).

CDK4 Gene Amplification

None of the osteosarcomas showed *CDK4* amplification.

RB LOH and pRb Immunohistochemistry

Twelve tumors (57%) (the 2 low-grade osteosarcomas and 10 high-grade osteosarcomas) exhibited nuclear immunohistochemical staining for pRb (Figure 2c). All five tumors that were immunonegative for p16 were immunopositive for pRb and showed two strong bands at the RB 1.20 polymorphism (Figure 3). Nine (47%) of the 19 high-grade osteosarcomas did not show any staining for pRb (Figure 2d).

Discussion

In this study, 4 of 19 high-grade osteosarcomas (3 osteoblastic and 1 giant cell-rich type) showed homozygous deletion of the *CDKN2A* gene and were immunonegative for p16 protein expression. An additional giant cell-rich osteosarcoma had immunonegative tumor cells but contained numerous immunopositive benign multinucleated giant cells. This tumor did not demonstrate ho-

mozygous deletion on comparative multiplex polymerase chain reaction analysis, presumably because the normal DNA from the numerous multinucleated cells obscured detection of the deletion. Alternatively, the gene may have been silenced in this tumor by *CDKN2A* promoter methylation, a mechanism that has been implicated in other human cancers. The close concordance between genetic and immunohistochemical results suggests that immunohistochemistry may be a rapid and reliable method for assessing the *CDKN2A*/p16 status in osteosarcomas. Thus, 5 of the 19 (26%) high-grade tumors had probable inactivation of the *CDKN2A* gene. This incidence is somewhat higher than previously reported in osteosarcomas, with other series reporting only 5–7% of osteosarcomas with *CDKN2A* deletions.^{27–29} The frequency of homozygous deletion in those studies may, however, be underestimated, because other studies either used Southern blotting,^{28,29} which may miss small deletions, or set comparative multiplex standards that would have missed cases with any contamination of normal tissue.²⁷

By immunohistochemical analysis, 9 of 19 (47%) high-grade osteosarcomas were immunonegative for pRb. This apparent rate of *RB* loss is in accordance with other studies that have shown up to 67% of osteosarcomas with *RB* mutations or gene loss.^{13,29} Significantly, however, all 5 tumors that had *CDKN2A*/p16 alterations showed two strong bands at the RB 1.20 locus and intact pRb expression, suggesting two intact copies of the *RB* gene in these osteosarcomas. In turn, all of the pRb-immunonegative tumors stained positively for p16. Thus, the p16-cdk4-pRb pathway is involved in a large number of high-grade osteosarcomas, with mutually exclusive p16 and pRb changes occurring in 14 of 19 (74%) high-grade tumors. Although none of our cases showed *CDK4* amplification, *CDK4* amplification has been demonstrated in a small number (9%) of osteosarcomas in another study,²⁸ again implicating this critical regulatory pathway in osteosarcoma oncogenesis. Furthermore, other components of this pathway, such as *CDK6*, need to be evaluated.

Osteosarcomas with *RB* alterations may have a more aggressive clinical course and a worse prognosis than osteosarcomas that lack *RB* loss^{13,30}; however, the response to preoperative chemotherapy does not appear to be affected by *RB* alterations.³⁰ *CDKN2A*/p16 alterations have also been postulated to affect prognosis adversely in patients with osteosarcoma,²⁷ with three patients whose tumors had *CDKN2A* deletions dying of disease within 34 months. Most of the patients in our study were recently diagnosed and have been followed for a limited time. However, two patients with high-grade osteosarcomas have died of their disease; one of them had *CDKN2A* deletion and the other, loss of pRb expression. Seven additional patients have developed metastatic disease (six to lungs and one to bones); five of these patients had *CDKN2A*/p16 (two) or *RB*/pRb alterations (three). Interestingly, an osteosarcoma that arose in a patient with Paget's disease of bone, which is known for its bad prognosis, had *CDKN2A*/p16 alterations. Although the number of patients in this study is too small

Figure 2. Immunohistochemistry for p16 (A, B, and E) and pRb (C, D, and F) (diaminobenzidine chromogen with hematoxylin counterstain). **A:** Diffuse nuclear and cytoplasmic staining for p16 in an osteosarcoma with an intact *CDKN24* gene. **B:** p16 immunonegativity in an osteosarcoma that showed homozygous deletion for the *CDKN24* gene. **C:** Strong nuclear staining for pRb in an osteosarcoma that was immunonegative for p16 and had retained both copies of the *RB* gene. **D:** Negative staining for pRb in an osteosarcoma that was immunopositive for p16. **E:** Giant cell-rich osteosarcoma showing no immunostaining for p16 of the malignant mononuclear cells, but diffuse staining of the benign multinucleated giant cells, which might explain the lack of homozygous deletion of the *CDKN24* gene in this tumor by comparative multiplex analysis. **F:** Same case as in (E) showing nuclear immunostaining for pRb of many malignant mononuclear cells but no staining of the multinucleated giant cells.

Figure 3. *RB* gene LOH. Allelic losses at the *RB* 1.20 polymorphism are noted in the osteosarcomas in lanes 4 and 5. Lanes 1–3 show two strong bands at the *RB* 1.20 polymorphism, suggesting two intact copies of the *RB* gene in these osteosarcomas. Lanes 4 and 5, however, show only faint lower bands, suggesting allelic loss of the *RB* gene in these two tumors; the two tumors were pRb immunonegative.

and the follow-up too short to draw any definite conclusions about the relationship between *CDKN2A*/p16 alterations and prognosis, the results indicate that molecular markers should be included in future studies of osteosarcoma response and survival.

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