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.

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) osteosarcomas.
sarcomas that arose from the posterior surface of the
distal femur. Nineteen tumors were high-grade (12 osteo-
brastic, 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 hu-
merus (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 re-
action 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 dele-
tions in tumors with less than 30% contaminating nonneo-
plastic cells.23

CDK4 Gene Amplification

CDK4 gene amplification was evaluated using a differen-
tial polymerase chain reaction assay.23,24 The products
were separated by electrophoresis on a 2% agarose gel, and visualized under
ultraviolet light with ethidium bromide staining. This assay
was used to detect homozygous CDKN2A dele-
tions in tumors with less than 30% contaminating nonneo-
plastic cells.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 anti-
body detects a single 16-kd band on Western blots of
human tissues, including brain tumors (J. Koh, unpublish-
data), and has been used in the immunohisto-
chemical evaluation of human brain tumors.26 Formalin-
fixed, paraffin-embedded tissues were sectioned at 6 μm
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 in-
cubated in a humidity chamber at room temperature for 2
hours. After the primary antibody incubation, a secondary
biotinylated horse anti-mouse antibody (Vector Laborato-
ries, Burlingame, CA) was applied at a 1:1000 dilution (in
1% bovine serum albumin/PBS) for 1 hour at room tem-
perature, followed by the avidin-biotin complex kit (ABC
Elite, Vector Laboratories) also for 1 hour at room tem-
perature. 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 counter-
stained in the hematoxylin solution Gill No. 1 (Sigma).
After dehydration in graded alcohols and clearing in xyl-
ene, the slides were coverslipped. Tonsil tissue served as
a control in which nuclear and cytoplasmic staining
was noted specifically in histiocytic cells in germinal cen-
ters and epithelial cells of the mucosal lining. Negative
controls were performed by omitting the primary antibody
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 incu-
bated with 10% normal horse serum in 1% bovine serum
albunin/PBS for 30 minutes. The primary mouse mono-
clonal anti-pRb antibody (G3–245, Pharmingen) was di-
luted 1:2500 and applied overnight at 4°C. The second-
ary 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 epi-
thelial layers.

Tissue for p16 and pRb immunohistochemical staining
was available from the original biopsy or resection spec-
imen in 18 cases; in three cases, the original biopsy
slides contained insufficient material for immunohisto-
chemistry, in which cases it was performed on the resec-
tion specimen after the patients had received preopera-
tive 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-
gous 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/HaeIII digest size marker, lanes 2: negative controls (no DNA lanes), Lane 3, top: normal control DNA.

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/HaeIII digest size marker, Lanes 2: negative controls (no DNA lanes), Lane 3, top: normal control DNA.

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 homozygous 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 bone); 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 CDKN2A gene. B: p16 immunonegativity in an osteosarcoma that showed homozygous deletion for the CDKN2A 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 CDKN2A 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.
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.

References

6. Horstmann MA, Posl M, Schoiz RB, Anderegg B, Simon P, Baumgaertl K, Deiling G, Kabitsch H: Frequent reduction or loss of DCC and RB gene LOH. Allelic losses at the RB 1.20 polymorphism are noted in these two tumors; the two tumors were pRb immunonegative.

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.