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p53 Protein and Proliferating Cell Nuclear Antigen (PCNA) Expression in Small Round Cell Tumors of Bone and Adjacent Soft Tissue

A Study of 60 Cases

Kenneth Devaney, M.D.,* Susan L. Abbondanzo, M.D.,†
Kris M. Shekitka, M.D.,‡ Robert B. Wolov, M.D.,†
and Donald E. Sweet, M.D.‡

Sixty small cell tumors of bone and adjacent soft tissue were studied in an attempt to define the incidence of immunohistochemically detectable p53 protein and correlate these findings with the results of proliferating cell nuclear antigen (PCNA) immunohistochemical staining and mitotic counts. All of the lesions had been formalin-fixed and paraffin-embedded; half were subjected to decalcification prior to processing. The study population included 12 Ewing's sarcomas of bone, 3 atypical Ewing's sarcomas of bone, 3 primitive neuroectodermal tumors of bone, 11 Askin tumors of the thoracopulmonary region, 11 small cell osteosarcomas of bone, 10 mesenchymal chondrosarcomas of bone, and 10 malignant lymphomas involving bone. The patients ranged in age at the time of presentation from 17 to 67 years. Overall, the incidence of p53 positivity was extremely low in these lesions, irrespective of tumor type. Positive nuclear staining with an antibody to p53 was found in none of the 12 Ewing's sarcomas, none of the 3 atypical Ewing's sarcomas, none of the 3 primitive neuroectodermal tumors of bone, 1 of the 11 Askin tumors of the thoracopulmonary region (1.5% of tumor cells positive), 1 of the 11 small cell osteosarcomas (2% of tumor cells positive), 1 of the 10 mesenchymal chondrosarcomas of bone (7% of tumor cells positive), and 2 of the 10 malignant lymphomas involving bone (0.5% and 1% of tumor cells positive, respectively). The majority of tumors showed PCNA positivity within the tumor cells, although the incidence of PCNA positivity within the histologic types varied greatly; in general, the higher PCNA counts corresponded to higher mitotic counts within the individual lesions. The present study did not demonstrate any correlation between mutant p53 accumulation detected by immunohistochemistry and tumor type, and so it is unlikely that p53 positivity will prove to be of great use in the differential diagnosis of these lesions. A correlation between p53 positivity and PCNA staining or mitotic activity was not apparent. *Int J Surg Pathol 2(4):259–268, 1995*

Key words: Ewing's sarcoma, Askin tumor, neuroectodermal tumor, mesenchymal chondrosarcoma, small cell osteosarcoma, lymphoma, p53, PCNA.

From *the Departments of Pathology and Orthopaedic Surgery, Brown University, Rhode Island Hospital, Providence, Rhode Island, and the Department of Pathology, University of Michigan, Ann Arbor, Michigan; †the Division of Immunohistochemistry, Armed Forces Institute of Pathology, Washington, DC; and ‡the Department of Orthopedic Pathology, Armed Forces Institute of Pathology, Washington, DC.

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Reprint requests: Kenneth Devaney, M.D., Department of Pathology 2G332/0054, University of Michigan, 1500 E Medical Center Drive, Ann Arbor, MI 48109-0054.

The introduction of new technologies into the practice of diagnostic pathology often invites a reexamination of what previously were challenging diagnostic problems in the hope of better defining diagnostic or prognostic criteria. The potential diagnostic dilemma presented by the "small round cell tumor of bone" is a classic example of this problem that is faced by the surgical pathologist in routine practice. Only recently, with the introduction of newer investigative modalities, such as antibodies directed against the HBA71 antigen and fluorescent *in situ* hybridization for detection of the cytogenetic abnormality t(11;22) for the prospective identification of the Ewing's sarcoma or primitive neuroectodermal tumor family lesions (often taken to include typical and atypical Ewing's sarcoma, the Askin tumor of the thoracopulmonary region, and primitive neuroectodermal tumors of bone and soft tissue), have great strides been made in addressing this vexing problem for the diagnostic pathologist.[1,2]

The present study was undertaken to determine the incidence of nuclear p53 protein positivity by immunohistochemical means in a large group of small cell bone lesions and to explore the potential application of these findings to the differential diagnosis and prognosis of these lesions. At the same time, proliferating cell nuclear antigen (PCNA) immunostaining of the lesions and calculation of the mitotic counts were carried out for correlation with the p53 results. In view of the ready availability of immunohistochemical techniques to the diagnostic surgical pathologist (and, conversely, the difficulty at present in carrying out sophisticated molecular studies in many hospitals), immunohistochemical staining for the presence of an antigen such as p53 might provide valuable prognostic, diagnostic, or therapeutic information, which may serve as a clinical assay for use in the general pathologist's practice.

Materials and Methods

Sixty biopsies of small cell lesions of bone and adjacent soft tissue were drawn from the consultation files of the Armed Forces Institute of Pathology and the Rhode Island Hospital. Twenty-nine of the cases were the subject, in part, of an earlier report of the immunohistochemical features of small round cell tumors of bone.[3] The remaining cases were collected both prospectively and from the archives of the Armed Forces Institute of Pathology and the Rhode Island Hospital; the oldest cases (embedded in paraffin) dated back to the 1960s.

In each instance, hematoxylin and eosin-stained sections were available for study; in many cases, periodic acid-Schiff-stained sections (both with and without diastase pretreatment) were available as well. Some of the lesions had been studied ultrastructurally, but this was not the focus of the present report and so was not uniformly employed in all of the study cases. The final diagnosis in each of the 60 study cases was based on the light microscopic features of the individual lesions, employing criteria that have been previously reported.[3]

The study population consisted of 12 classic osseous Ewing's sarcomas (Fig. 1), 3 atypical osseous Ewing's sarcomas, 3 primitive neuroectodermal tumors of bone (Fig. 2), 11 Askin tumors of the thoracopulmonary region (Fig. 3), 11 small cell osteosarcomas of bone (Fig. 4), 10 mesenchymal chondrosarcomas of bone (Fig. 5), and 10 malignant lymphomas involving bone. The patients ranged in age at the time of presentation from 17 to 67 years.

All tissues studied by immunohistochemistry had been formalin-fixed and paraffin-embedded; approximately half had been subjected to decalcification prior to processing (by a variety of methods). Five of the Ewing's sarcomas, 5 of the mesenchymal chondrosarcomas, 4 of the Askin tumors, 3 of the small cell osteosarcomas, and 4 of the lymphomas consisted of lesions in which both decalcified and undecalcified tumor blocks were available; in these 21 tumors, a comparison between decalcified and undecalcified specimens with regard to differences in the incidence of either p53 or PCNA immunostaining was performed. The antibody to p53 was a polyclonal antibody to mutant type p53 (CM1, Novocastra Laboratories, Newcastle upon Tyne; diluted 1:400), while the antibody to PCNA was a

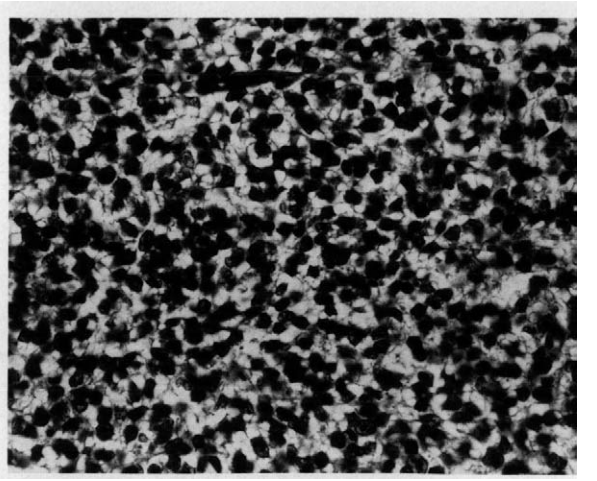

Fig. 1. The classic Ewing's sarcoma is marked by a sheetlike proliferation of relatively uniform small round cells, often with clear to finely vacuolated cytoplasm and small nuclear chromocenters (hematoxylin and eosin, original magnification ×220).

Fig. 2. Peripheral neuroectodermal tumor of bone was defined by light microscopy as a lesion composed of sheets of cells similar to those of Ewing's sarcoma but with the added architectural requirement that more than half of the tissue showed evidence of pseudorosette formation (formation of true rosettes was not noted) (hematoxylin and eosin, original magnification ×120).

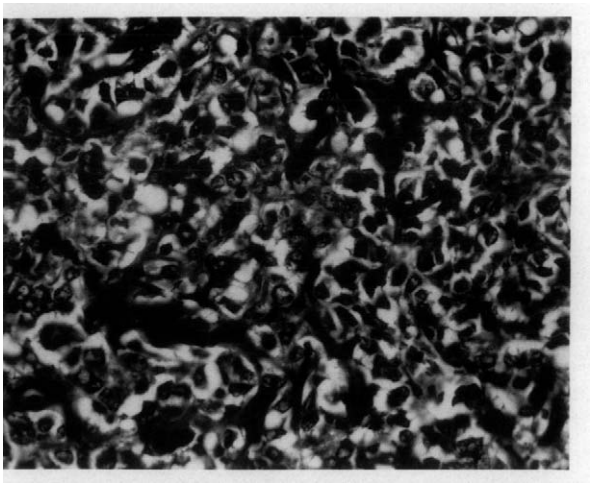

Fig. 4. The small cell osteosarcomas were characterized by a proliferation of hyperchromatic round to oval tumor cells that often exhibited a greater degree of nuclear pleomorphism than was seen in the classic Ewing's sarcomas; formation of osteoid by the tumor cells (as seen here) was found only focally (hematoxylin and eosin, original magnification ×220).

monoclonal preparation (Dako Corp., Carpenteria, CA; diluted 1:500). Sections were developed using a modified avidin-biotin-peroxidase complex technique.[4] Controls used within each run included positive and negative control tissues; formalin-fixed, paraffin-embedded colonic adenocarcinoma served as the positive control for p53 staining (over 90% of the tumor cells were consistently positive), while negative controls consisted of a section of each tumor being studied with substitution of nonimmune rabbit serum for the primary antibody. Antigen retrieval was carried out by heating deparaffinized tissue sections for 10 minutes in a solution of citrate buffer. A microwave oven was employed as a source of heating for this process.

In each case, 200 tumor cells were counted, and the presence or absence of positive nuclear staining with antibody to p53 and to PCNA was noted. In cases in which a variable degree of staining was appreciated within the specimen, counting was done in the most intensely stained zone. The percentage of positive cells was recorded in each

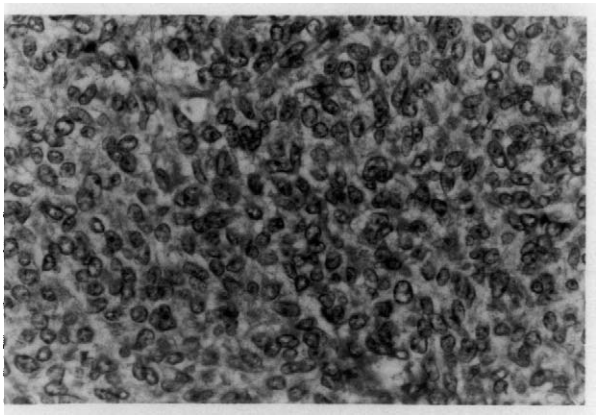

Fig. 3. The component cells of the Askin tumors of the thoracopulmonary region resembled Ewing's sarcoma cells in many lesions, with a somewhat greater tendency toward elongation of the nuclei in some areas (hematoxylin and eosin, original magnification ×220).

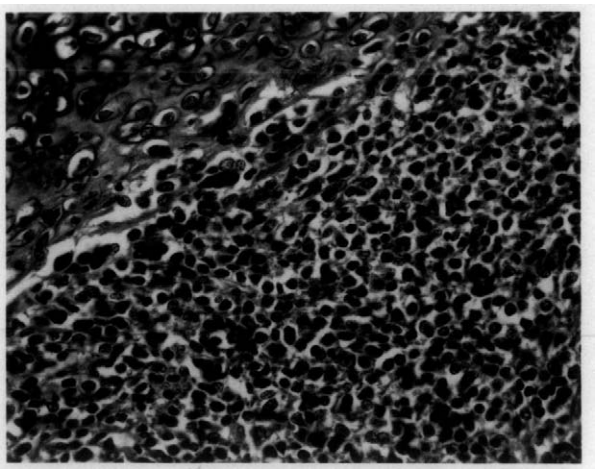

Fig. 5. A juxtaposition of two components (islands of cartilage embedded in a sea of hyperchromatic small round to oval cells) typified the mesenchymal chondrosarcomas of bone (hematoxylin and eosin, original magnification ×220).

instance. Within the same region, the number of mitotic figures was counted in 20 high-power fields (HPF) (Olympus microscope, 40× objective and 10× ocular lens, field area 0.159 mm^2) and recorded as mitoses per 10 HPF.

Results

Positive nuclear staining with an antibody to p53 was found in none of the 12 Ewing's sarcomas, none of the 3 atypical Ewing's sarcomas, none of the 3 primitive neuroectodermal tumors of bone, 1 of the 11 Askin tumors of the thoracopulmonary region (1.5% of tumor cells positive), 1 of the 11 small cell osteosarcomas (2% of tumor cells positive) (Fig. 6), 1 of the 10 mesenchymal chondrosarcomas of bone (7% of tumor cells positive) (Fig. 7), and 2 of the 10 malignant lymphomas involving bone (0.5% and 1% of tumor cells positive, respectively).

Some degree of PCNA positivity was found in the majority of tumors studied, irrespective of the histologic subtype; however, within each histologic type, a wide variation in the percentage of positive cells was seen. Specifically, 10 of 12 Ewing's sarcomas were PCNA positive (range, 1% to 92% of tumor cells positive, mean 29%) (Fig. 8); 2 of 3 atypical Ewing's sarcomas were PCNA positive (5% and 94% of tumor cells positive, respectively); 2 of 3 primitive neuroectodermal tumors of bone were PCNA positive (1% and 13% of tumor cells positive, respectively); 11 of 11 Askin tumors of the thoracopulmonary region were PCNA positive (range, 12% to 94% of tumor cells positive, mean 43%); 10 of 11 small cell osteosarcomas were PCNA positive (range, 1% to 88% of tumor cells positive, mean 34%); 9 of 10 mesenchymal chondrosarcomas of bone were PCNA positive (range, 2% to 66% of tumor cells positive, mean 19%); and 9 of 10 malignant lymphomas involving bone were PCNA positive (range, 1% to 87% of tumor cells positive, mean 41%).

The highly variable degree of PCNA positivity within the individual tumor types corresponded to the variable mitotic count within the lesional types studied. The Ewing's sarcomas of bone had from 1 to 18 mitoses/10 HPF; the atypical Ewing's sarcomas had from 2 to 11 mitoses/10 HPF; the primitive neuroectodermal tumors had from 1 to 7 mitoses/10

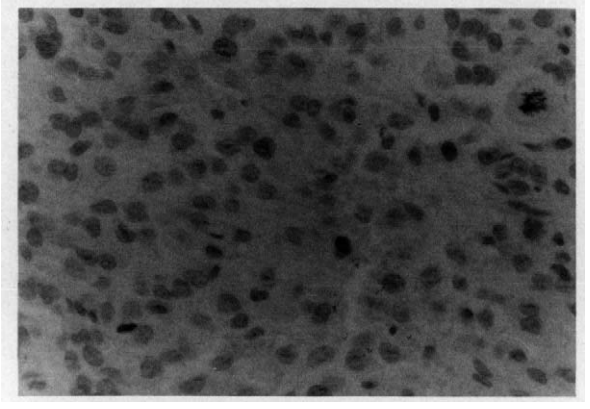

Fig. 6. Immunostaining of this small cell osteosarcoma with antibody to p53 yielded only rare positive cells (center of figure) (antibody to p53, hematoxylin counterstain, original magnification ×220).

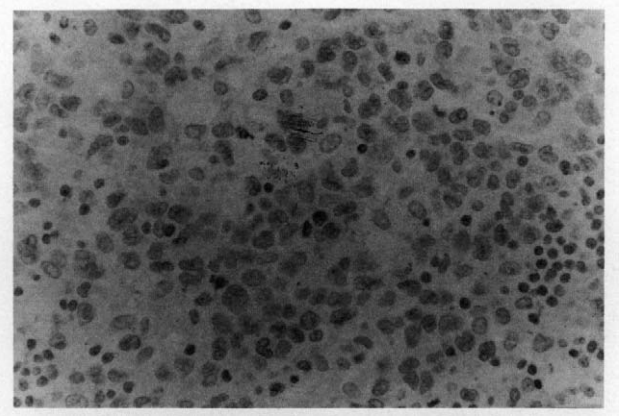

Fig. 7. Immunostaining of this malignant lymphoma of bone with antibody to p53 yielded only rare positive tumor cells (antibody to p53, hematoxylin counterstain, original magnification ×220).

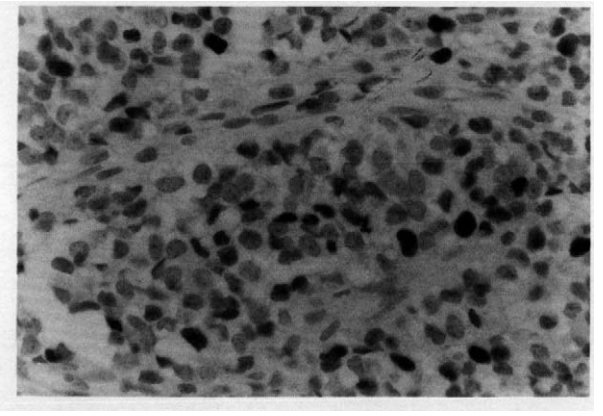

Fig. 8. Immunostaining with antibody to proliferating cell nuclear antigen (PCNA) yielded some degree of positive nuclear staining in the majority of the tumors studied, although the proportion of positive cells varied greatly from one lesion to another; shown here is a classic Ewing's sarcoma of bone in which approximately 35% of the tumor cells were PCNA positive (antibody to PCNA, original magnification ×220).

HPF; the Askin tumors of the thoracopulmonary region had from 2 to 28 mitoses/10 HPF; the small cell osteosarcomas had from 3 to 38 mitoses/10 HPF; the mesenchymal chondrosarcomas of bone had from 1 to 9 mitoses/10 HPF; and the malignant lymphomas involving bone had from 4 to 18 mitoses/10 HPF.

A direct correspondence between an increasing level of PCNA positivity and the mitotic count was noted in this study; this correspondence (that is, the finding of a high percentage of PCNA positive tumor cells in a given tumor usually predicted a higher mitotic rate within that lesion) was found irrespective of the histologic group of tumor studied.

No relationship was seen between the rare p53 positive tumors in each group and either the percentage of PCNA positive cells or the mitotic counts; that is, p53 positivity did not correspond either to a strikingly high or strikingly low rate of PCNA positivity or mitotic activity.

Previous decalcification did not produce an appreciable effect on the incidence or quantity of PCNA staining. The impact of prior decalcification on p53 immunostaining was more difficult to judge, in view of the extremely low incidence of p53 staining found in the study population overall.

Discussion

The present study found a consistently low incidence of positive nuclear staining of tumor cells with antibody to mutant p53 in a variety of small cell lesions of bone, including the Ewing's/primitive neuroectodermal family of tumors, small cell osteosarcoma, and mesenchymal chondrosarcoma. To place these observations in context, Table 1 includes a tabulation of selected reports on the incidence of positive immunohistochemical staining of tumor cell nuclei in a variety of musculoskeletal tumors as well as the findings of the present investigation.[5-13]

It has been established that, among the tumor suppressor genes that have been characterized to date, abnormalities involving the p53 gene (also known as the TP53 gene) are extremely common in a host of different human tumor types.[14-30] The normal p53 gene (located on the short arm of chromosome 17) plays a role in the regulation of a cell's entry into the S phase, producing a nuclear phosphoprotein (the wild-type p53 protein) that acts to interfere with the transcription of DNA and so impedes cell proliferation. In experimental systems, the wild-type p53 protein has been shown to slow or even inhibit altogether cellular transformation by oncogenes.[18] Inactivation of the p53 gene may result either from deletion of the gene or from its mutation. The precise mechanisms are extremely complex and continue to be incompletely understood; however, it is known that a variety of different mutations may affect the p53 gene in different tumor types.[17,18] The phenomenon of p53 alteration has been best studied in large intestinal adenocarcinomas, in which a majority of tumors exhibit a loss of one p53 allele via mutation and a loss of the other p53 allele through deletion.[17,18]

Table 1. Frequency of p53 Positivity by Immunohistochemistry in Musculoskeletal Tumors

Tumor Type	Location	Frequency of p53 Positivity
Osteosarcoma (not further subclassified)	Bone	Common
Small cell osteosarcoma	Bone	Rare
Chondrosarcoma (not further subclassified)	Bone	Uncommon
Mesenchymal chondrosarcoma	Bone	Rare
Malignant fibrous histiocytoma	Bone	Rare
Giant cell tumor	Bone	Rare/uncommon
Ewing's sarcoma/PNET family tumors	Bone	Rare
Malignant fibrous histiocytoma	Soft tissue	Uncommon
Atypical fibroxanthoma	Soft tissue	Common
Liposarcoma	Soft tissue	Uncommon
Rhabdomyosarcoma	Soft tissue	Common
Leiomyosarcoma	Soft tissue	Common
Malignant peripheral nerve sheath tumor	Soft tissue	Common
Adult fibrosarcoma	Soft tissue	Rare/uncommon
Congenital/infantile fibrosarcoma	Soft tissue	Common
Fibromatosis	Soft tissue	Rare
Dermatofibrosarcoma protuberans	Soft tissue	Rare
Nodular fasciitis	Soft tissue	Rare
Malignant mesothelioma	Soft tissue	Common
Angiosarcoma	Soft tissue	Common
Pyogenic granuloma	Soft tissue	Common

PNET, peripheral primitive neuroectodermal tumor; rare, reported in less than 10% of tumors of this type; uncommon, reported in 10% to 30% of tumors of this type; common, reported in more than 30% of tumors of this type.

With regard to the present study, the failure of an appreciable number of the small cell osteosarcomas to stain with antibody to the p53 protein is somewhat at odds with the expected results as might have been predicted by the reports describing the finding of relatively frequent mutations of the p53 gene in osteosarcomas with accompanying p53 protein accumulation by immunohistochemistry, as noted in Table 1.[6,7] This may simply reflect the occasional lack of close correlation between these two parameters, as has been hinted at in other tumor types. It has been noted in some tumors, including some hematopoietic and hepatic neoplasms, that the incidence of p53 protein overexpression may exceed the incidence of p53 gene mutations.[31–33] Such an apparent discrepancy may indicate accumulation of a nonmutated p53 protein (particularly likely when the antibody employed recognizes epitopes on both wild-type and mutant p53 protein), or even cross-reactivity of the antibody with other nuclear components than the p53 protein itself.

Moreover, despite a finding of p53 mutation (as detected by polymerase chain reaction) in some sarcomas, the corresponding production of a protein detectable by immunohistochemistry is not always observed.[7] Such an occurrence may reflect the presence of a p53 gene mutation that produces not a missense mutation, which typically yields a detectable increase in nuclear p53 protein by immunohistochemistry, but that results in a nonsense mutation or even a gross deletion of the gene. In addition, failure of immunohistochemistry to detect p53 protein accumulation may correspond to technical inadequacies within the tissue, such as over- or underfixation. Others have observed that, while p53 mutations are usually not found in primary tumor samples of Ewing's sarcoma, cell lines derived from those same tumors often will show evidence of p53 gene mutation, reflecting, perhaps, some survival advantage conferred in the culture environment by the mutation.[34,35]

Such observations raise questions about the reliability of p53 immunostaining as a marker for detectable mutations of the p53 gene in at least some mesenchymal tumors. It may prove to be that (in contrast to many carcinomas) some sarcomas either do not show frequent p53 gene mutations, or these mutations may fail to result in the production of a protein product not detectable by presently employed techniques. To date, mutations of the p53 gene have been identified less frequently in sarcomas than in epithelial malignancies; among the two mesenchymal tumor types that do exhibit an appreciable frequency of p53 mutation by gene sequencing are rhabdomyosarcomas and osteosarcomas.[19,36,37] Apparent overexpression of the p53 protein product (manifested as positive immunohistochemical staining) has been reported in an admixture of non-neoplastic, benign, and malignant soft tissue lesions, suggesting that further studies are required to explain these findings.[9]

The present investigation employed a widely used antibody directed toward mutant p53 protein, the CM1 polyclonal antibody. As in all immunohistochemical investigations, our study is subject to variations that may have been produced by a number of potential variables, including type of tissue fixative employed, length of fixative exposure, and varying sensitivities and specificities among the various commercially available antisera. The potential influence of these and other variables must be borne in mind when interpreting the results of an immunohistochemical study such as ours. Antigen retrieval was employed in an attempt at minimizing the impact of these potentially confounding factors.

This study of paraffin-embedded tissues did not permit either comparison of frozen with fixed tissue sections or of decalcified with undecalcified tissues within the same tumor in each instance. Five of the Ewing's sarcomas, 5 of the mesenchymal chondrosarcomas, 4 of the Askin tumors, 3 of the small cell osteosarcomas, and 4 of the lymphomas consisted of lesions in which a portion of the tumor had been decalcified and a portion had not. In these 21 tumors, a comparison between decalcified and undecalcified specimens did not yield an appreciable difference in the incidence of either p53 or PCNA immunostaining, suggesting that decalcification does not appear to have an appreciable effect on these results.

Although an estimation of a tumor's mitotic activity has classically been cited as an index of that tumor's proliferative activity (and hence, presumably, provides some insight into the degree of aggressiveness of that tumor as an independent histologic parameter), it has been suggested by some observers that this is not necessarily always the case. Mitosis counting has been criticized as lacking in reproducibility among different (and even the same) observers, subject to variation as a function of tissue processing, and simply lacking significance in strict follow-up studies of certain tumor types.[38–46]

In light of the above considerations, attempts have been made at identifying alternate methods of assessing proliferative activity, methods that would prove to be both reproducible and statistically significant. These alternative approaches may be divided into two broad groups: first, the more technically difficult or expensive methods, which

include the use of flow cytometry, tritiated thymidine, and bromodeoxyuridine; and second, the less involved/or expensive methods, including immunohistochemical staining for Ki-67 and PCNA. With regard to PCNA staining in particular, some significance for PCNA staining (either as a diagnostic or as a prognostic tool) has been reported in such disparate lesions as hepatocellular tumors, epithelial-myoepithelial carcinoma of salivary gland origin, mast cell disease, endometrial adenocarcinoma, renal carcinoma, synovial sarcoma, and dysplastic bronchial epithelium.[47–54]

As was the case with p53 immunostaining, questions have arisen with regard to the specificity of the various antibody preparations available for identifying PCNA, as well as the potentially confounding influences that may be provided by such factors as type and length of fixation employed and other technical variables.[55–63] In general, PCNA labeling increases as the mitotic count within the tissue section increases.[64] This was seen in our series of small cell tumors of bone as well. Separate groups of lesions (distinct, that is, from groups that might have been delineated based on mitotic activity alone) were not distinguished by PCNA staining results.

The present study did not demonstrate any correlation between nuclear p53 protein accumulation as detected by immunostaining and tumor type, and so it is unlikely that p53 positivity will prove to be of great use in the differential diagnosis of these lesions. A correlation between p53 positivity and PCNA staining or mitotic activity was likewise not apparent. While follow-up data on our material is limited, the rare p53 positive tumors did not appear to exhibit an unusually aggressive or unusually indolent course when compared with the p53 negative lesions.

Among the avenues open for future exploration is the relationship of the protein product of the murine double minute-2 gene (MDM2 gene located on the long arm of chromosome 12) to p53 gene mutation. Some have suggested that MDM2 amplification may inactivate the same regulatory pathway as does p53 mutation. It appears that these two mechanisms may act, at least in mesenchymal tumors, exclusively of one another, that is, preliminary findings suggest that a given sarcoma may exhibit either p53 mutation or MDM2 amplification but usually not both.[65–67] It has been suggested that those infrequent sarcomas exhibiting both p53 mutation and MDM2 amplification may follow a more aggressive course than those harboring only one of these abnormalities.[66] Confirmation of these observations promises to yield further insights into the genesis and progression of tumors of mesenchymal tissues.

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