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Widespread p53 Overexpression in Human Malignant Tumors

An Immunohistochemical Study Using Methacarn-fixed, Embedded Tissue

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p53 is a nuclear protein believed to play an important role, through mutation and overexpression, in the progression of human malignant tumors. The authors employed a monoclonal antibody, 1801, and investigated overexpression of p53 in a series of 255 malignant and benign tumors, using deparaffinized sections of methacarn-fixed tissue. Overall, immunohistochemically detected p53 overexpression was found in 39% of malignant tumors, with considerable variation within individual tumor types (34% of breast carcinomas, 92% of ovarian carcinomas, 33% of soft tissue sarcomas). Homogenous, heterogenous, and focal immunostaining patterns were noted. With rare exceptions, no immunostaining of any benign tumors was noted. No immunostaining was found in adjacent, benign tissues, or in a series of fetal tissues. This is the first demonstration of widespread p53 overexpression in alcohol-fixed, embedded tissue and confirms the major role played by p53 in human malignancies. (Am J Pathol 1992, 140:145–153)

The nuclear phosphoprotein p53 was first identified as a host protein complexed with simian virus 40 (SV40) large T antigen, the major transforming protein of the virus.[1] Although originally considered an oncogene, evidence now suggests that p53 acts as a tumor suppressor gene in its wild-type form, with only mutated forms manifesting tumorigenic activity.[2–4] *In vitro*, the wild-type p53 gene product can reduce the transforming efficiency of many oncogenes and can suppress tumor growth.[5] The mutant forms of p53 participate in cell immortalization and cooperate with other cellular oncogenes such as *ras* in the malignant transformation of various cell lines.[3,4,6] The p53 gene, located on the short arm of chromosome 17, is a common site of mutation or deletion in human tumors, including those of breast, colon, lung, liver, mesenchyme, bladder, and myeloid origins.[7–15] In addition, germ line mutations of p53 have been detected in individuals with Li-Fraumeni syndrome, a familial syndrome characterized by the occurrence of multiple tumors.[16] Little is known, however, about the role of p53 in the progression of most human tumors *in vivo*. Other investigators have found that immunohistochemical detection of p53 is associated with the presence of mutated p53 alleles.[7,17] Immunohistochemical analysis thus far has been restricted largely to frozen tissue and has shown increased levels of p53 in malignant tumors of breast, colon, and lung.[17–20] We now demonstrate the utility of monoclonal anti-p53 antibody 1801 in methacarn-fixed, deparaffinized tissue and characterize expression of the protein in human malignant and benign tumors, normal adult tissue, and fetal tissue.

Materials and Methods

Cases

Two hundred fifty-five blocks of methacarn-fixed benign and malignant tumors acquired between 1984 and 1991 were obtained from the archives of the Department of Pathology at the University of Washington Medical Center, Seattle, Washington. A wide spectrum of tumors were thus obtained, including those primary to: breast (54), ovary (19), lung (34), gastrointestinal tract (19), kidney (5), soft tissue (42), uterus (4), bladder (6), lymph node

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(17), central nervous system (28), and endocrine system (15) (Table 1). Comparably fixed normal tissue corresponding to these same sites was also examined. In addition, corresponding snap-frozen tissue that had been stored at −70°C was available in nine of the tumor cases. p53 overexpression also was examined in tissues obtained from five fetuses (gestational ages ranging from 54 to 137 days). In all cases, tissue had been fixed in methacarn for 4 to 24 hours at room temperature, processed, and embedded in paraffin.

Controls

For control studies, we generated frozen and methacarn-fixed and paraffin-embedded cell pellets of a SV40-transformed human fibroblast cell line (PSV-811; American Type Culture Collection [ATCC]), which overexpresses mutated p53 (as a positive control) and a human sarcoma cell line (Saos-2; ATCC), which lacks both p53 alleles (as a negative control).[21]

Immunohistochemistry

An anti-human p53 murine monoclonal antibody, 1801, developed by Banks et al,[22] was generously sent to us in supernatant form by Dr. Crawford. Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method, as previously described.[23] An optimal dilution of 1:100 of the supernatant on deparaffinized, methacarn-fixed tissues was determined by titration experiments. Tissue was incubated with the primary antibody for 1 hour; biotinylated anti-mouse gamma G immunoglobulin (IgG; Vector Laboratories, Burlingame, CA) was applied at a dilution of 1:1000 for 30 minutes followed by streptavidin peroxidase (Jackson Immunonuclear Laboratories, Westgrove, PA) at 1:5000 for an additional 30 minutes. Slides were developed using 3,3′-diaminobenzidine in 0.1 mol/l (molar) TRIS buffer, pH 7.6, with the nickel chloride color enhancement method as previously described.[24] As a control for tissue immunoreactivity, each case was also immunostained with 19A2, an anti-PCNA (proliferating cell nuclear antigen) monoclonal antibody (Coulter Laboratories).

Results

Validation of Antibody Immunostaining in Deparaffinized Tissues

In deparaffinized tissue blocks made from methacarn-fixed cell pellets, reactivity with the anti-p53 antibody was confined to the cell nucleus. In SV40-transformed human fibroblasts (PSV-811), known to overexpress a mutated form of p53, 100% of cell nuclei were positive; no immunostaining was noted in the p53-negative Saos-2 cell line (Figure 1A). In cell mixing experiments, the fraction of 1801-positive cell nuclei corresponded well with the fraction of PSV-811 cells (Figure 1B, C). There were no qualitative or quantitative differences between the anti-p53 immunostaining pattern in these deparaffinized preparations and parallel frozen sections made from the same cell pellets (data not shown). Furthermore in those nine cases in which methacarn-fixed, paraffin-embedded, and frozen samples of human tumors were available, no immunostaining differences were noted (Figure 2).

Analysis of Anti-53 Immunostaining in Human Tissues and Tumors

In all positive cases, p53 overexpression was confined to the tumor cell population: when present, the cells of the tumor stroma, including proliferating cell compartments such as inflammatory cells and blood vessels, were negative (Figure 3). All normal tissue adjacent to tumors was negative.

No anti-p53 immunoreactivity was noted in any of the 50 benign tumors (Figure 4), with the exception of one ovarian adenofibroma, which had a solitary focus of adenocarcinoma in a different portion of the specimen, and a single leiomyoma, which was histologically unremarkable. Both p53-positive benign tumors contained only very rare positive nuclei. No p53 overexpression was found in any of the sets of fetal tissues (n = 5) studied. All of the latter displayed excellent reactivity with the anti-PCNA antibody (Figure 5).

Of the malignant tumors studied (n = 208; Table 1), anti-p53 immunoreactivity was present in 82, or 39%. Whereas cases were considered positive if any of the tumor cells manifested nuclear positivity, three major patterns of p53 positivity, based on the percentage of positive tumor cells, were evident: a group with virtually all the tumor cells positive (Figure 3H–J); a group with a heterogenous immunostaining pattern, with approximately 30% to 70% of tumor cells positive (Figure 3K, L); and a group with less than 5% of tumor cells positive (Figure 3M, N). The pattern of staining in all but five of the positive cases was exclusively nuclear. In those five cases (three breast and two small cell lung carcinomas), both nuclear and cytoplasmic staining were present (Figure 6B). Although there were insufficient case numbers in tumor subgroups to examine for significance, there did not appear to be any correlation between tissue or tumor type and the pattern of anti-p53 immunostaining.

As summarized in Table 1, the frequency of anti-p53

Table 1. *Frequency of Anti-p53 Immunoreactivity*

Tumor	n	1801 # reactive (%)
Breast		
Malignant		
Infiltrating ductal	24	9
Metastatic ductal	11	6
DCIS	7	2
Infiltrating lobular	3	0
LCIS	1	0
Subtotal	46	17 (37)
Benign		
Fibroadenoma	6	0
Lactating adenoma	2	0
Ovary		
Malignant		
Adenocarcinoma	9	8
Mixed Mullerian	4	4
Granulosa cell	2	0
Subtotal	15	12 (80)
Benign		
Adenofibroma	4	1*
Lung		
Small cell CA	10	6
Adenocarcinoma	13	7
Squamous cell CA	7	4
Mesothelioma	3	0
Carcinoid	1	0
Subtotal	34	17 (50)
Gastrointestinal		
Colon carcinoma	9	4
Gallbladder carcinoma	4	3
Neuroendocrine/carcinoid	6	0
Subtotal	19	7 (37)
Kidney		
Renal cell carcinoma	5	3 (60)
Soft tissue		
Malignant		
Leiomyosarcoma	9	2
Rhabdomyosarcoma	2	0
Liposarcoma	1	0
Primative neural tumor	1	1
Ewing sarcoma	6	3
Chondrosarcoma	1	1
MFH	3	0
Malignant NST	2	0
Pleomorphic sarcoma	2	2†
Subtotal	27	9 (33)
Benign		
Leiomyoma	8	1 (12)
Hemangiopericytoma	4	0
Fibroma	3	0
Endocrine		
Malignant		
Adrenal carcinoma	2	1 (50)
Pheochromocytoma	1	0
Follicular carcinoma	4	0
Benign		
Follicular adenoma	7	0
Oxyphilic adenoma	1	0
Bladder		
Transitional cell carcinoma	5	1
Squamous cell carcinoma	1	1
Subtotal	6	2 (33)
Lymph		
Malignant		
B cell	10	3
T cell	2	1
Hodgkins	5	1

Table 1. (*Continued*).

Subtotal	17	5 (29)
Benign		
Reactive lymph node	3	0
Central nervous system		
Glioma-grades 1 and 2	7	4
Glioblastoma multiforme	16	5
Subtotal	23	9 (39)
Schwannoma	2	1 (50)
Chordoma	2	0
Thymus		
Benign thymoma	1	0
Malignant thymoma	1	1 (100)
Uterus		
Adenocarcinoma	4	1 (25)
Total	208	82 (39)

* Focus of adenocarcinoma identified in separate block.
† Rare large pleomorphic cells positive.

immunoreactivity varied greatly among the various tumor sites: 34% of breast, 92% of ovarian, 50% of lung, 44% of colon, 60% of renal carcinomas, and 33% of all soft tissue sarcomas were positive. In the latter cases, p53 overexpression was noted in only selected histologic types; the findings in individual tumor sites and histologic types are listed in Table 1.

Although total numbers of individual tumor types were too few to permit statistical analysis, there did not appear to be a significant correlation between anti-p53 immunoreactivity and morphologic tumor grade. For example, 57% of grade I or grade II gliomas and 31% of glioblastoma multiforme were positive. In the breast carcinomas examined, there was no apparent correlation between tumor nuclear grade and the presence of p53 overexpression, although 54% of breast carcinomas examined from various metastatic sites were p53-positive, compared with 37% from primary tumors in the breast.

Discussion

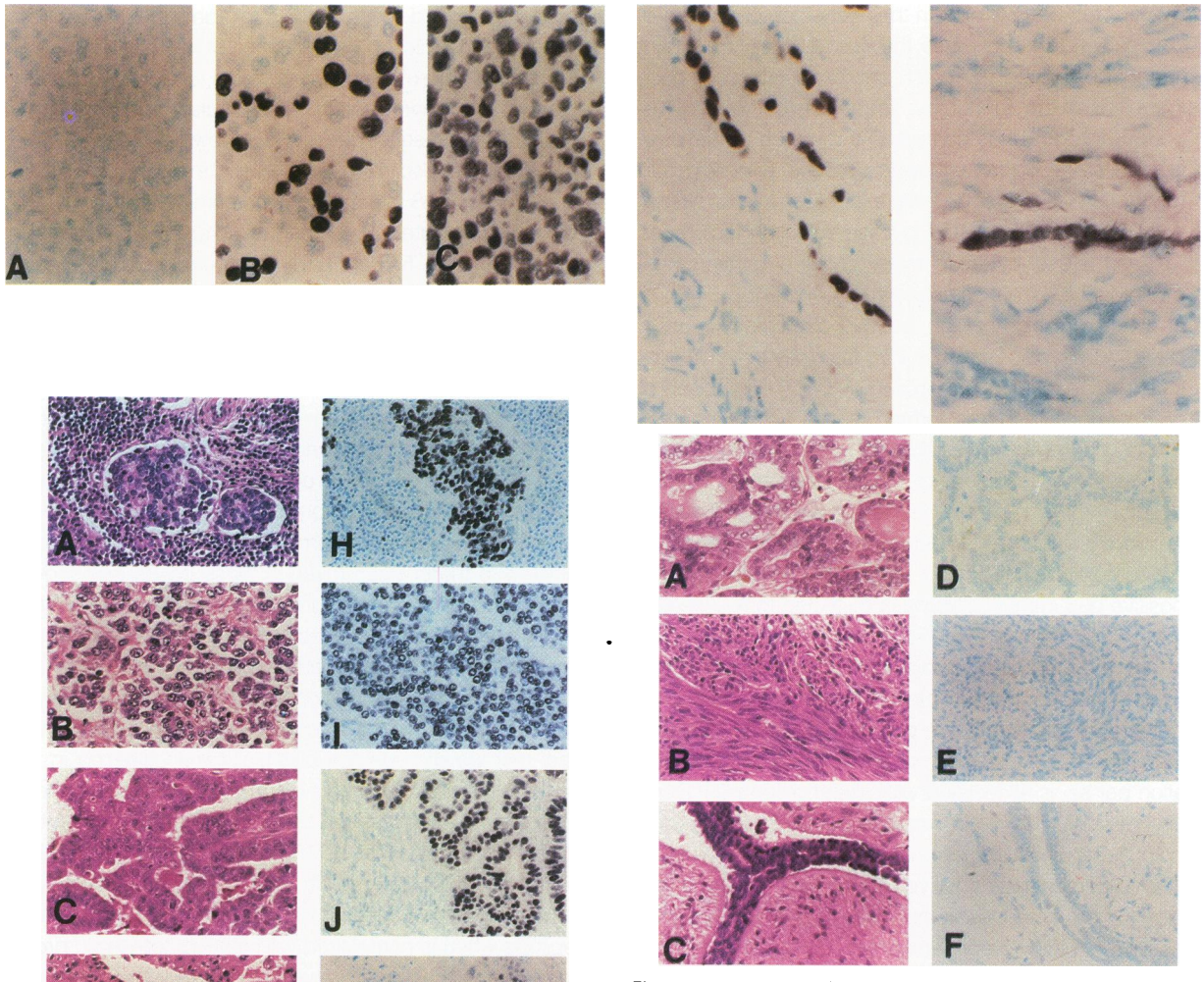
We have identified p53 overexpression at the protein level using immunohistochemical techniques in a wide range of human malignant tumors with an anti-p53 monoclonal antibody; this represents the first comprehensive demonstration of anti-p53 immunoreactivity using fixed, embedded tissue specimens, and also represents the first demonstration of p53 overexpression in endometrial, renal, and adrenal carcinomas, and the first demonstration of p53 overexpression by immunohistochemical methods in ovarian carcinomas, bladder carcinomas, malignant lymphomas, gliomas, and various sarcomas. Previous immunohistochemical studies of p53 overexpression in human tumor specimens have largely employed frozen sections; the use of fixed embedded material permits superior histologic preservation as well as the use of retrospective material. We have been unable to obtain comparable immunostaining on deparaffinized, formalin-fixed tissue; thus these studies add further support for the use of alcohol-based fixatives such as methacarn, which have been previously documented to be superior for preservation of other antigens such as intermediate filaments proteins.[23]

Previous work suggests that immunohistochemical or immunoblotting detection of p53 protein is possible only if there is overexpression of mutant p53.[7,17,18,22] In our study, no anti-p53 immunoreactivity was noted in normal tissues. Recent immunohistochemical studies using antibodies to various epitopes of p53 have identified high levels of p53 in frozen sections of breast, lung, and colon carcinomas. Cattoretti et al[19] found 15.5% of infiltrating breast carcinomas positive using anti-p53 antibody pAb421 and 45.5% positive using antibody 1801. Antibody pAb421 recognizes mutant and wild-type, murine and human p53; it reacts with a denaturation-resistant epitope located between amino acids 370 and 378.[22] Although the reason for the discrepancy of staining between the two antibodies was not known, p53 overexpression identified by either antibody was associated with estrogen-receptor–negative, epidermal growth factor receptor–positive, high nuclear grade tumors. Iggo et al[17] recently examined 40 lung carcinomas using anti-p53 antibody pAb240 and found p53 overexpression in 82% of squamous cell carcinomas, 57% of adenocarcinomas and 44% of small cell carcinomas. Carcinoid tumors and adjacent normal epithelium in all of their cases were negative. pAb240 recognizes human and murine p53 in its mutant forms only. DNA sequencing of four of the lung tumors confirmed that mutations were present in the cases that overexpressed the protein and absent in a carcinoid tumor that did not overexpress the protein. Expression of p53 in colon carcinoma and benign adenomas of the colon was examined immunohistochemically

by Van den Berg et al.[20] In their series of 29 colon carcinomas and 74 adenomas, they found 55% of malignant tumors positive with pAb421, and dysplastic cells positive in 8% of the adenomas. The results of the study reported here are thus comparable to much of the published data using frozen tissue sections. Differences in reported frequency of p53 overexpression in some tumor types may be attributable to case selection factors, as well as the use, by these investigators, of different anti-p53 monoclonal antibodies (eg, 421 and 240).

The expression of high levels of p53 clearly is associated with transformation *in vitro* and may be an indicator of malignant transformation *in vivo*.[25–28] High levels of p53 accumulate in transformed murine and human cell lines[29]; p53 overexpression also has been identified in high levels in a wide range of murine primary tumors, suggesting that p53 may be used as a tumor cell marker in mice.[30] The data presented here, as well as those previously published, thus support the hypothesis that positive p53 immunostaining is restricted to a subset of malignant tumors. p53 mutation and overexpression may be the most common genetic alteration in the development of human malignancies.[17,31] The lack of immunohistochemically detectable p53 in fetal tissues suggests that malignant transformation, and not increased proliferation per se, is associated with alterations in p53. Demonstration of positive p53 immunostaining thus may be of potential diagnostic significance. In general, we were unable to demonstrate positive p53 immunostaining in benign tumors examined in this study (Table 1). Rare p53-positive nuclei were observed in one case of a leiomyoma and one case of a benign ovarian tumor, however, of the more than 50 nonmalignant tissues examined in this study. It is possible that the presence of rare p53-positive cells in these tissues suggests the presence of a potentially malignant subpopulation; indeed, in the one ovarian tumor, there was a malignant subpopulation elsewhere in the specimen. This would be consistent with the proposed role of p53 mutation as one of a sequence of mutational events leading to the development of malignancy, as documented in carcinoma of the colon.[32] In a subset of colonic adenomas, which are considered premalignant lesions, p53 mutation and overexpression also have been found[20]; we also have found positive p53 immunostaining in cases of severe dysplasia within the squamous epithelium of the oral cavity.[33] It also may be possible that p53 overexpression is a reversible phenomenon; the description by Tadini et al[34] of anti-p53 immunostaining in the nuclei of basal cells of psoriatic skin may be evidence of this phenomenon. In a related study of head and neck tumors, we have observed positive p53 immunostaining confined to the periphery of nests of well-differentiated squamous cell carcinoma, corresponding to the zone of cell proliferation in these tumors.[33] It will be critical to extend these studies to additional tissues to assess the validity and significance of these findings.

In all but five of the positive tumors, antibody localization was exclusively nuclear. Both mutant and wild-type p53 may be present in the nucleus; however, mutant p53 may be found also in the cytoplasm.[35] In transformed cell lines, mutant p53 forms oligomeric complexes with other cytoplasmic proteins such as the SV40 large T antigen, adenovirus type 5 58-kd E1b, and members of the heat shock 70 protein family, greatly enhancing its stability and half-life.[35,36] Although the presence of p53 in the cytoplasm clearly identifies the protein as an abnormal form, immunohistochemically detected protein in the nucleus theoretically could represent wild-type or mutant protein. Cytoplasmic immunostaining was reported by Iggo et al,[17] using pAb240 in small cell carcinomas of lung; in the remainder of the immunohistochemical studies cited above, however, p53 immunostaining has been restricted to the nucleus (even in those cases that show mutation by DNA sequencing). Considering that normal levels of p53 are undetectable by immunohistochemical methods and the normal protein has a very short half-life, it is most likely that monoclonal antibody 1801 is identifying mutant protein in the nucleus of the tumor cells. The fact that we did see cytoplasmic immunostaining in a small number of the tumors examined, and that a higher frequency of cytoplasmic staining has been seen with pAb240, suggests that the 1801 antibody may be unable to reliably detect the protein when it is in the cytoplasm. This may be because of decreased availability of the epitope when is complexed with other cytoplasmic proteins, or the presence of levels of protein below that detectable by immunocytchemical techniques using antibody 1801. The significance of the malignant tumors lacking immunohistochemically detectable p53 is uncertain. Wild-type p53 is thought to be involved in control of DNA transcription by some as yet unidentified mechanisms[31,37,38] loss of this wild-type function, either through mutation or allelic loss, is thus associated with malignant transformation.[14,37] Mutant p53 can complex with the wild-type protein, an action that may prevent DNA transcription, or may have some other inhibitory effect on the wild-type protein. Mutated forms also appear to have direct oncogenic activity unrelated to inhibition of normal p53 function.[20] The tumors in our study with immunohistochemically undetectable p53 could represent tumors with 'normal' levels of wild-type p53, tumors overexpressing mutant p53 protein not identified by the antibodies or immunohistochemical techniques employed, or tumors that have deleted both alleles, resulting in no p53 protein expression. To further distinguish among these groups, in future prospective studies we will measure mRNA levels by Northern blotting and *in situ* hybridization; deletion of both alleles, for example, will result in absence of



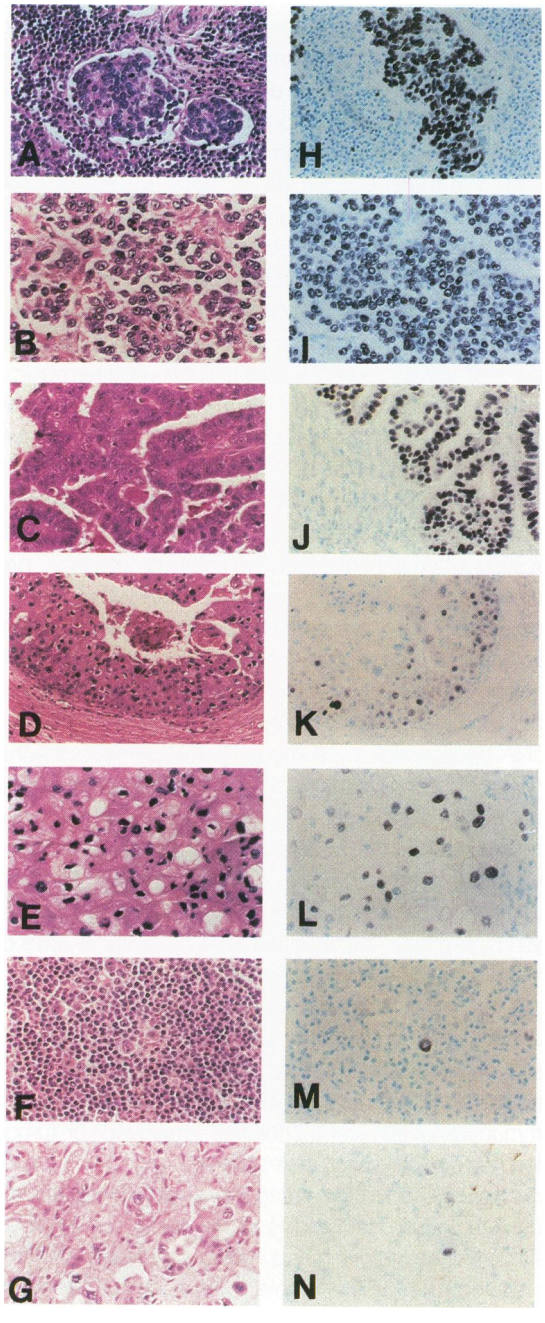


Figure 1. *Anti-p53 antibody immunostaining of cell blocks of admixtures of Saos-2 cells (a cell line that lacks native p53) and PSV-811 cells (a transformed cell line that overexpresses mutant p53). Shown are methacarn-fixed cell pellets that contain either 100% Saos-2 cells* (A), *50% Saos-2 and 50% PSV-811 cells* (B), *or 100% PSV-811 cells* (C). *Streptavidin-biotin immunoperoxidase method; original magnification ×400.*

Figure 2. *Deparaffinized sections of methacarn-fixed* (A) *and corresponding acteone-fixed, frozen section* (B) *of infiltrating ductal breast carcinoma immunostained with anti-p53 antibody 1801. The intensity and pattern, (nuclear and cytoplasmic) of staining of the methacarn-fixed tissue are comparable to that of the frozen. Streptavidin-biotin immunoperoxidase method; original magnification ×400* (A,B).

Figure 3. *Deparaffinized sections of methacarn-fixed malignant tumors stained with hematoxylin and eosin* (A–G) *and immunostained with antibody 1801* (H–M) *demonstrating different patterns of positive nuclear immunostaining. Tumors represented are: metastatic cell carcinoma* (A,H); *Ewing's sarcoma* (B,I); *ovarian papillary serous adenocarcinoma* (C,J); *ductal carcinoma* in situ *of breast* (D,K); *renal cell carcinoma* (E,L); *Hodgkin's lymphoma* (F,M); *lung adenocarcinoma* (G,N). *Note three different immunostaining patterns: one in which virtually all tumor nuclei are positive* (H–J), *one in which there is heterogeneous immunostaining of tumor nuclei* (K,L), *and one in which only rare tumor nuclei are positive* (M,N). *Streptavidin-biotin immunoperoxidase technique; original magnification ×200* (A–N).

Figure 4. *Deparafinized sections of methacarn-fixed benign tumors stained with hematoxylin and eosin* (A–C) *and immunostained with anti-p53 antibody 1801* (D–F). *Tumors represented are: follicular adenoma of thyroid* (A,D); *leiomyoma of uterus* (B,E); *fibroadenoma of breast* (C,F). *There is no immunostaining of any of the sections. Streptavidin-biotin immunoperoxidase technique; original magnification ×200* (A–F).

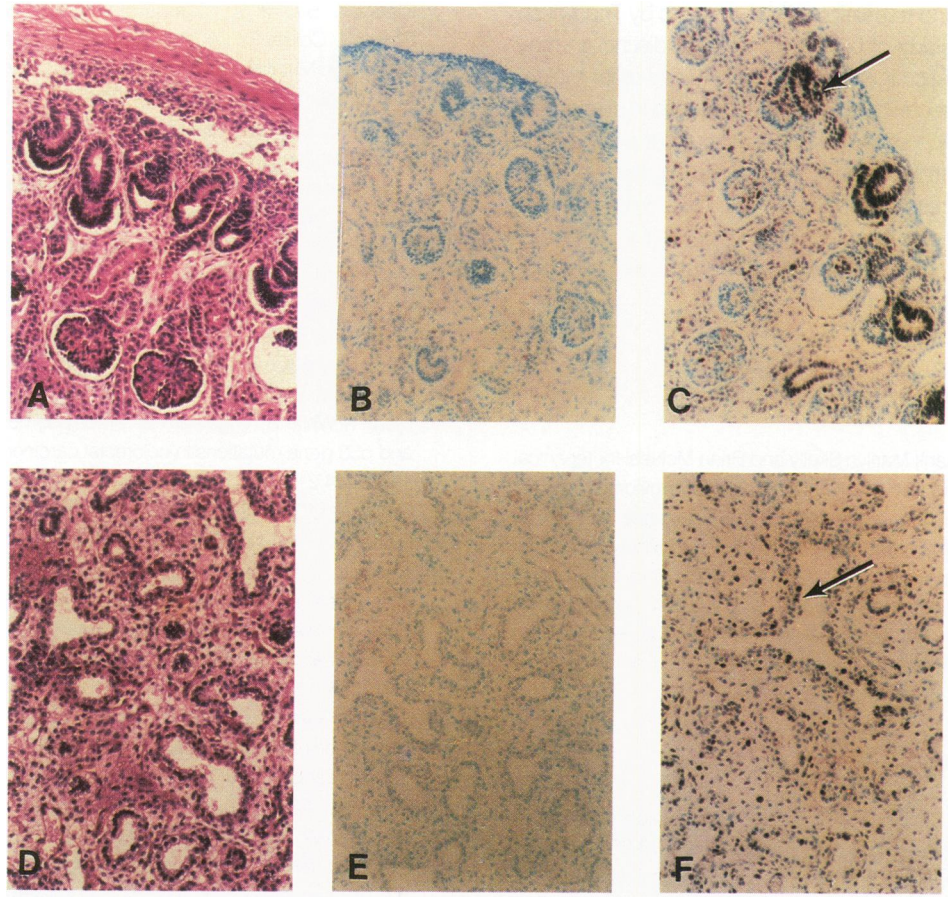


Figure 5. *Deparaffinized sections of methacarn-fixed kidney* (A–C) *and lung* (D–F) *from a 137 day fetus. Hematoxylin and eosin-stained sections* (A,D) *of kidney and lung, respectively, with corresponding sections immunostained with anti-p53 antibody 1801* (B,E) *and anti-PCNA antibody 19A2* (C,F). *Note complete absence of anti-p53 immunostaining* (B,E) *despite abundant nuclear reactivity with the anti-PCNA antibody, especially in the developing glomerular structures* (C, *arrow*) *and lung epithelium* (F, *arrow*). *Streptavidin-biotin immunoperoxidase technique; original magnification ×200* (A–F).

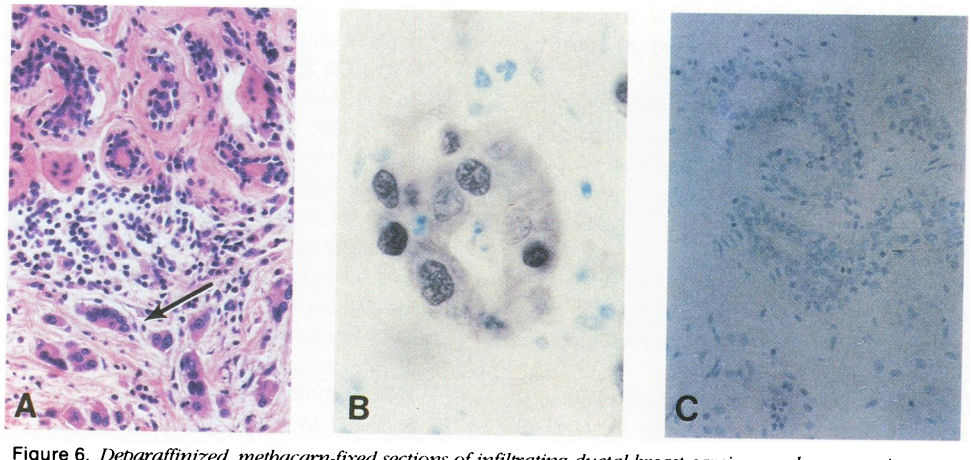


Figure 6. *Deparaffinized, methacarn-fixed sections of infiltrating ductal breast carcinoma demonstrating positive immunostaining with antibody 1801.* A: *H&E stained section of the carcinoma (arrow) and adjacent normal ducts.* B: *Area of carcinoma showing p53 immunolocalization to the nucleus as well as the cytoplasm of the carcinoma cells.* C: *A section of normal breast tissue taken from this patient's contralateral breast is negative with the anti-p53 antibody.*

p53 protein and transcripts. As suggested by Thompson et al,[39] in a study of breast cancer, this distinction may have prognostic significance. Use of these other molecular biologic techniques also will be of help in elucidating the significance of the different patterns of p53 overexpression noted in our study. For example, heterogeneous p53 overexpression may be a function of wild-type p53 expression in a subset of the tumor cells or a function of malignant progression resulting in allelic loss in a subpopulation of the tumor cells.

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