Mutations in the *p53* Gene in Pulmonary Blastomas:

Immunohistochemical and Molecular Studies

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Well-differentiated fetal adenocarcinomas and biphasic blastomas are types of lung cancer that contain glands that mimic the appearance of fetal lung. Biphasic blastomas also show a primitive embryonic stroma. Despite histological similarities leading these two tumors to be classified as pulmonary blastomas, they have distinct clinical and prognostic features. Little information is available on genetic changes in these tumors because they are rare; therefore, the authors studied nine biphasic blastomas and 12 well-differentiated fetal adenocarcinomas for the presence of mutations in the *p53* gene. Mutations in the *p53* gene are common in other lung cancers, and the type of mutation in the *p53* gene can provide information about the original or inciting mutagens. The authors found five biphasic blastomas (42%) had mutations in the *p53* gene by immunohistochemical and molecular analysis, whereas none of the well-differentiated fetal adenocarcinomas contained mutations. These results provide molecular support for the significance of distinguishing between well-differentiated fetal adenocarcinoma and biphasic blastoma histologically and identify several types of *p53* gene mutations that occur in these tumors. HUM PATHOL 1117–1123. This is a US government work. There are no restrictions on its use.

Key words: **pulmonary blastoma, cancer, lung, *p53* gene, tobacco smoke.**

Abbreviations: **BB, biphasic blastoma; WDFA, well-differentiated fetal adenocarcinoma; PCR, polymerase chain reaction; H&E, hematoxylin and eosin; SSCP, single-strand conformation polymorphism analysis; ABC, avidin-biotin complex; TE, tris(hydroxymethyl)aminomethane and ethylenediaminetetra acetic acid.**

Pulmonary blastomas are neoplasms composed of malignant glands or mesenchyme that microscopically resemble fetal lung in the pseudoglandular phase of lung development.[1] Pulmonary blastomas were initially recognized as biphasic tumors, but in 1982, Kradin et al and associates reported a histological variant containing only fetal-type glands and tubules without a malignant stroma.[2] They referred to this tumor as pulmonary endodermal tumor resembling fetal lung; however, the authors have used the alternative term *well-differentiated fetal adenocarcinoma* (WDFA) when referring to this neoplasm.[1,2] Study of the clinical and pathological features of these tumors show that at presentation biphasic neoplasms (biphasic blastomas [BBs]) are significantly larger radiographically, more likely to be symptomatic, and are more frequently accompanied by pleural effusions than WDFAs.[1] Histologically, the BBs are mitotically active and can contain bizarre tumor giant cells. More important, the prognosis of biphasic blastomas is significantly worse than that of WDFA, with 10-year survival of about 20% for the former and about 80% for the latter.[1]

Pulmonary blastomas are rare, and molecular and cytogenetic abnormalities have not yet been described in these tumors. It is known that despite the name blastoma, which suggests a childhood tumor, these tumors occur largely in adults, and 82% of the patients with this malignancy are tobacco smokers.[1] These features suggest that pulmonary blastomas might show genetic mutations similar to those found in other lung cancers. Specifically, mutation of the *p53* gene is common in human tumors[3] including lung cancer.[4-6] In addition, the mutational spectrum of the *p53* gene in lung cancer may reflect specific mutagens, such as tobacco smoke, causing the malignancy.[7,8] For this reason, the authors examined pulmonary blastomas for *p53* gene mutations.

The authors used two methods in this study to screen for and characterize *p53* gene mutations: immunohistochemistry and molecular analysis. Immunohistochemistry has been used to identify elevated expression of the protein product of the *p53* gene, which is associated with *p53* gene mutations. It is useful as a screening technique to identify *p53* gene mutations in some types of cancer including lung cancers,[9,10] but it does not identify all mutations.[4,11,12] Further, in some malignancies, immunohistochemical staining for p53 protein does not seem to correlate well with the presence of mutations.[13,14] Molecular analysis can be time-consuming, but it yields information about the specific sequence abnormality, which is useful in implicating mutagens. It is also capable of identifying some mutations that cannot be found with immunohistochemistry.[4] Because of this, the authors used both immunohistochemical and molecular analysis to assure fullest

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identification of *p53* gene mutations and to characterize the nucleic acid sequence of the mutations found. The techniques used for the molecular analysis of *p53* gene mutations were designed to yield accurate information using the small amounts of paraffin-embedded, formalin-fixed tissues available for study in these patients. The authors used molecular screening with single-strand conformational polymorphism analysis (SSCP); sequencing of a small region of the gene was then used to confirm and characterize an SSCP result suggesting mutation. All sequences chosen for amplification were less than 500 nucleotides in length, and mutations were sought in only exons 5 to 9. Studies have shown that mutations in the *p53* gene occur throughout the entire length of the *p53* gene in lung cancer[4,15]; however, most mutations occur in exons 5 to 8. Therefore, the authors predicted that examining this area would yield most of the *p53* gene mutations in these tumors.

MATERIALS AND METHODS

Specimens

Cases diagnosed as pulmonary blastoma with available paraffin blocks were obtained from the pathology archives of the Department of Pulmonary and Mediastinal Pathology of the Armed Forces Institute of Pathology. The tumors included 12 biphasic blastomas and 9 WDFA, and represent a subset of a larger series whose clinical and pathological features have been reported.[1] Briefly, tumors classified as BB contain both malignant glands and stroma resembling embryonic lung. WDFA is a tumor composed of malignant fetal-type glands and benign stroma. The specimens selected all contained a large fragment of nonnecrotic tumor.

Immunohistochemistry

Immunohistochemical staining for p53 protein was performed after antigenic enhancement (microwaving) with a cocktail of D07 and D01 antibodies obtained from Oncogene Science (Uniondale, NY) and Novocastra Laboratories (Newcastle-upon-Tyne, UK) at the suggested concentrations, using an avidin-biotin complex (ABC) procedure with Vectastain reagents (Burlingame, CA). This cocktail was chosen by comparison with pAb 1801, (Oncogene Science, San Diego, CA),[16] CM-1, (Novocastra Laboratories) DO7 or DO1 antibody staining alone because the cocktail gave the most intense nuclear staining in tumor tissue, without cytoplasmic or stromal staining in nontumor tissue. Staining for Ki67 was performed with MIB-1 antibody (Amac Inc, Westbrook, ME) at suggested concentrations. Staining was evaluated by extent (none, <10%, 10% to 50%, and >50% of the tumor cells), and intensity (negative, mild, moderate, and strong)

DNA Extraction

Tumor in three 50-μm sections cut from formalin-fixed, paraffin-embedded tissue was separated from normal lung tissue by comparison with adjacent routine hematoxylin-eosin (H&E) stained sections. The tumor tissue was scraped with a scalpel into a microfuge tube, immersed in 1 mL of xylene, centrifuged in a microcentrifuge at 12,000*g* for 5 seconds, and the xylene was decanted. The residual tissue was rinsed twice in 100% ethanol, then desiccated. Desiccated tissue was immersed in 100 to 250 μL of TE (10 mmol/L tris(hydroxymethyl)aminomethane–chloride, pH 7.4; and 1 mmol/L ethylenediaminetetra-acetic acid) containing 50 μg/mL of proteinase K and 1% nonylphenoxy polyethoxy ethanol (NP40), then incubated for 24 to 48 hours, replenishing proteinase K at 5-hour intervals. After incubation, the mixture was extracted with phenol, chloroform, precipitated with sodium acetate, and resuspended in TE. DNA was separately extracted for sequencing and SSCP.

TABLE 1. Primer Pairs

Exon 5	Outer	5′ CTGCCGTGTTCCAGTTGC
		3′ TCAGTGAGGAATCAGAGGCC
	Inner	5′ TTATCTGTTCACTTGTGCCCTGACT
		3′ ACCCTGGGCAACCAGCCCTGTCGTC
Exon 6	Outer	5′ CTGGAGAGACGAACAGGGCTG
		3′ TTAACCCCTCCTCCCAGAGA
	Inner	5′ GCCTCTGATTCCTCACTGAT
		3′ GATCAAGCTTCCAGAGACCCCAGTTGCAAAC
Exon 7	Outer	5′ CTTGCCACAGGTCTCCCCAA
		3′ TCAGCGGCAAGCAGAGGCTG
	Inner	5′ GCGCACTGGCCTCATCTT
		3′ TGTGCAGGGTGGCAAGTGGC
Exon 8 & 9	Outer	5′ TTGGGAGTAGATGGAGCCT
		3′ GGCATTTTGAGTGTTAGACT
	Inner	5′ TTCCTTACTGCCTCTTGCTT
		3′ CATCGAATTCTGGAAACTTTCCACTTGGAT

PCR Amplification

PCR amplification for SSCP and sequencing was performed using standard buffers (Perkin-Elmer, Norwalk, CT), and 2.5 mmol/L nucleotides (Pharmacia, Piscataway, NJ). Nested amplification of exons 5 to 9 were performed with nested primer pairs (Table 1) at 1 μmol/L concentrations. The PCR amplification conditions were 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; 72°C for 10 minutes; and a 4°C soak. A few microliters of the primary amplification reaction were diluted 1:25, and 1 mL was used to inoculate the secondary nested amplification.

SSCP

Nested labeled PCR products were denatured and electrophoresed as reported previously.[4,17,18]

Sequencing

Nested PCR reaction products were separated and purified from low-melt agarose gels. Direct sequencing was performed using Sequenase version 2 enzyme (US Biochemicals, Arlington, IL) and the original amplification primers.[4,17] Mutations found by SSCP were verified by sequencing a separate PCR reaction.

RESULTS

SSCP and Sequencing

All 21 tumors were screened for mutations by SSCP in exons 5 to 9 (see "PCR Amplification" and Table 1), and 5 cases with potential mutations were identified; 3 with SSCP abnormalities in exon 5, one in exon 7, and one in exon 8 (Table 2; Fig 1). Sequencing showed that all of the tumors with abnormal SSCP contained missense mutations in the *p53* gene in the exon indicated by SSCP analysis (Table 2; Fig. 2). All five patients with *p53* gene mutations in their tumor had biphasic

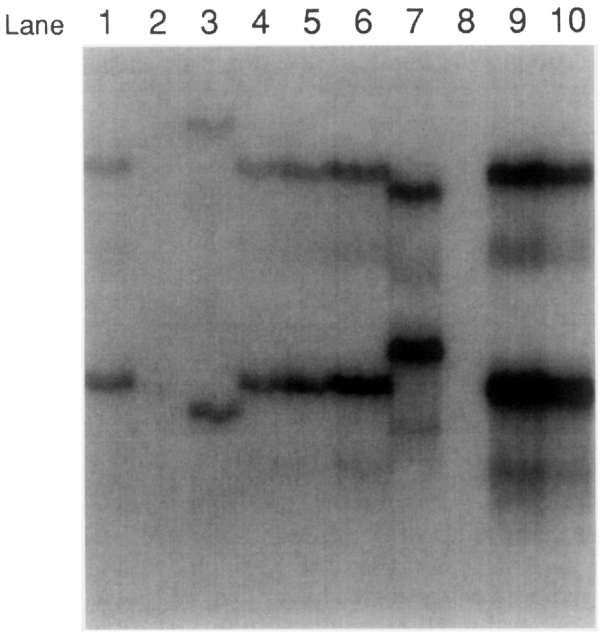

FIGURE 1. Exon 5 SSCP of 8 tumors: Lane 1 is a wild-type control using placental DNA; lane 2 is a negative control using no DNA, and mutations observed in lanes 3 and 7 correspond to tumors one and five (Table 2), respectively. In lane 8, there was no amplification on this PCR-SSCP run, a problem that occurred occasionally with paraffin-embedded formalin-fixed DNA but rarely with cell line or tumor DNA. Repeat PCR/SSCP showed wild-type bands in this tumor. Lanes 4, 5, 6, 9, and 10 give no evidence of mutation in exon 5 in these tumors.

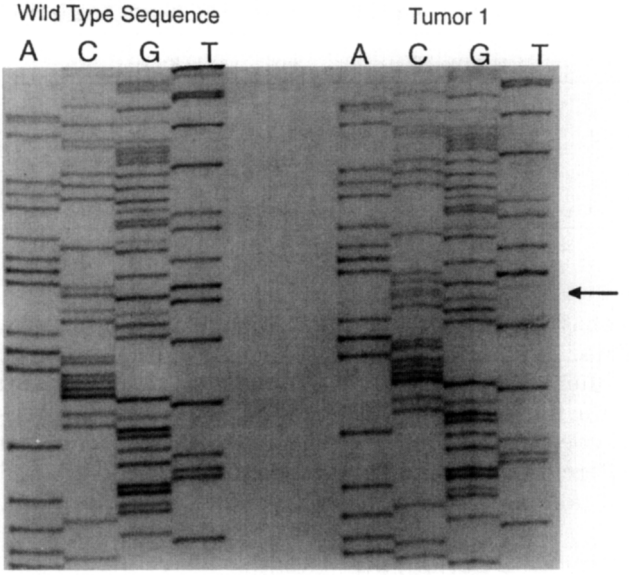

FIGURE 2. Sequencing gel of exon 5. This sequence from tumor one (Table 2) reveals a mutation in codon 182 (*) in which the wild-type TGC is mutated to a CGC sequence in this tumor. This nucleotide transition creates a cysteine to arginine change in the predicted mutant protein. A small amount of wild-type sequence can be noted in the background of the mutant sequence, and may represent noncarcinomatous stromal cells or infiltrating inflammatory cells incompletely separated from tumor tissue.

blastomas (5 of 12, or 42%), and the pertinent histological features and clinical histories of these patients are noted in Tables 2 and 3. None of the tumors from patients with WDFA contained *p53* gene mutations; therefore, these results show that *p53* gene mutations are statistically more frequent in biphasic blastomas than in well-differentiated fetal adenocarcinomas ($P = .045$ by Fisher's exact test).[19] Of the five tumors with *p53* gene mutations suggested by SSCP and confirmed by sequencing, three contained transition mutations, and two contained transversion mutations (Table 2; Figs 1 and 2). Of the three tumors containing transition mutations, two of the transitions were located at CpG dinucleotides, and one tumor contained a double transition within the same codon, producing a single amino acid change (Table 2; Fig 2). One of the transversions that was identified was a G:C to T:A transversion typical of benzpyrene exposure. The patient with this mutation had a heavy smoking history, and a smoking history was present in all of the patients with mutations (Table 3).

In some of the SSCP and sequencing gels, a slight background band typical of the wild-type allele was noted superimposed on the darker mutant pattern of bands (Fig 2). In all five of the cases showing molecular evidence of mutation of *p53* gene mutation by SSCP and sequencing, examination of the corresponding H& E slides revealed infiltration of inflammatory cells and invaginations of stroma into the tumor. In these cases, the predominance of a single mutant band in both SSCP and sequencing gels suggested the presence of a single clonal population in the tumor with a slight admixture of normal (unmutated) inflammatory or stromal cells.

More detailed histological review of the 21 cases showed that tumor giant cells (nuclei more than five times larger than a lymphocyte; Table 2) were found in 5

TABLE 2. Histological and Molecular Findings in Cases With *p53* Mutations

Case No.	Histology	Tumor Giant Cells	Staining Region	IHC %	IHC Intensity	SSCP (Exon No.)	Sequencing Data
1	BB	Present	Mainly epithelial	50	2+	5	TGC → CGC transition codon 182
2	BB	Present	Stromal and epithelial	70	2+	7	TGT → TTT transversion codon 238
3	BB	Present	Stromal > epithelial	60	1+	8	CGT → TGT transition codon 273
4	BB	Absent	Epithelial > stromal	90	3+	5	CCG → ACG transversion codon 152
5	BB	Present	Stromal > epithelial	55	2+-3+	5	GCC → ACT double-transition codon 161

Abbreviations: IHC, immunohistochemistry; SSCP, single-strand conformation polymorphism analysis.

TABLE 3. Clinical Characteristics of the Five Cases With *p53* Gene Mutation

Case No.	Age (Yr)	Sex	Race	Smoking History	Clinical Course
1	33	M	Black	yes	alive and well at 43 months
2	58	M	Black	yes, "heavy"	died of tumor at one month
3	48	M	White	NA	died of unknown causes at 305 mos
4	59	F	NA	yes, 1.5 to 2 packs for many years	alive and well at 86 months
5	39	F	White	yes, 7 pack per year history	died of tumor at 11 months

biphasic blastomas, 4 of which contained *p53* mutations. Thus, *p53* mutations were associated with the presence of tumor giant cells in these tumors ($P = .004$).[20] Two of the five patients with biphasic blastoma and mutations survived (Table 3). There was no statistically significant difference in survival between the patients who did and did not have demonstrable *p53* mutations.

Immunohistochemical Findings

Immunohistochemical staining for p53 protein was present in 11 cases and was exclusively nuclear (Figs 3 and 4). Scattered nuclear staining (<10% of tumor cells) was noted in six tumors that did not contain mutations by molecular analysis. In contrast, five neoplasms showed staining of greater than 50% of tumor cells, and all of these tumors contained mutations shown by SSCP and sequencing. Selected clinical and histological features of these cases are noted in Table 2. In the five biphasic tumors with staining on >50% of tumor cells, immunostaining was found in both epithelial and stromal components of the tumor, although staining could be predominantly in one or the other portion of the tumor (Table 2; Fig 3). The intensity of staining was not a helpful criterion for identifying tumors with molecular evidence of mutation because one tumor with a mutation showed only light diffuse staining, whereas strongly positive staining in less than 10% of nuclei was noted in six tumors that lacked mutations by molecular analysis.

Staining for Ki67 occurred in 12 of the tumors, and in all cases was present in less than 10% of cells and was always found in a scattered distribution. In five tumors without mutations, scattered Ki67 immunostaining was noted in the same areas as scattered p53 protein staining of individual cells (Fig 2). Squamous morules, previously noted in histological studies of these tumors, were the sites for scattered staining of both p53 and Ki67 in three of these tumors.

DISCUSSION

These studies show that mutations in the *p53* gene are found in pulmonary blastomas. These results provide the first evidence of a molecular abnormality in these tumors. Molecular events may influence the different histological and clinical presentations of the tumor because these results show that *p53* gene mutations are statistically more frequent in biphasic blastomas, a type of blastoma that is associated with a poorer prognosis than WDFA.[1] Furthermore, the finding of nuclear staining for p53 protein in both stromal and epithelial elements of biphasic blastomas and the molecular evidence of a single identifiable mutation in those tumor with *p53* gene mutations support the clonal nature of the tumors, despite the biphasic histological appearance.

Association of mutations in the *p53* gene with the histological type of pulmonary blastoma is consistent with findings in other tumors wherein *p53* gene mutations may be associated with a specific type or subtype of tumor. For example, Burkitt's lymphoma contains *p53* gene mutations more often than many other lymphoid malignancies, whereas some tumors, such as thyroid tumors, do not tend to contain *p53* gene mutations.[21,22] The frequency of *p53* gene mutations in biphasic blastomas is similar to the frequency of *p53* gene mutations in other lung cancers;[8] however, the lack of *p53* gene mutations in WDFA suggests that the favorable prognosis of WDFA may be because of lesser numbers of molecular changes in these tumors. Other histological features are also related to *p53* gene mutations because the presence of *p53* gene mutations in pulmonary blastomas was statistically associated with the presence of tumor giant cells.

In this study, the authors have defined criteria relating immunohistochemical staining for p53 protein to mutations in the *p53* gene, specifically nuclear staining of greater than 50% of tumor cells. The criteria defined herein for immunohistochemical screening for p53 mutations in pulmonary blastoma may not pertain in other types of tumors. For example, diffuse positive staining may characterize other lung cancers with *p53* gene mutations,[23] whereas staining for p53 protein may not be associated with *p53* gene mutations in some lymphomas.[13] The reasons for these differences are unclear, but it does suggest that the use of immunohistochemistry to screen for mutations in the *p53* gene may require empiric evaluation in a specific type of tumor. The authors also identified staining for p53 protein, which was not associated with *p53* gene mutations by molecular analysis. It was found in a scattered distribution in less than 10% of tumor cells. The authors postulate that p53 protein staining unassociated with the presence of a *p53* gene mutation may be related to changes in the level of p53 protein during the cell cycle because in this study it was found in the same areas and pattern as staining with Ki67, and in other tumors[13] and in vitro,[24] such a relationship has been postulated.

Biphasic tumors represent an intriguing paradox. Although cancer is generally considered a clonal disease, biphasic tumors show both epithelial and stromal differentiation. These results suggest that despite their

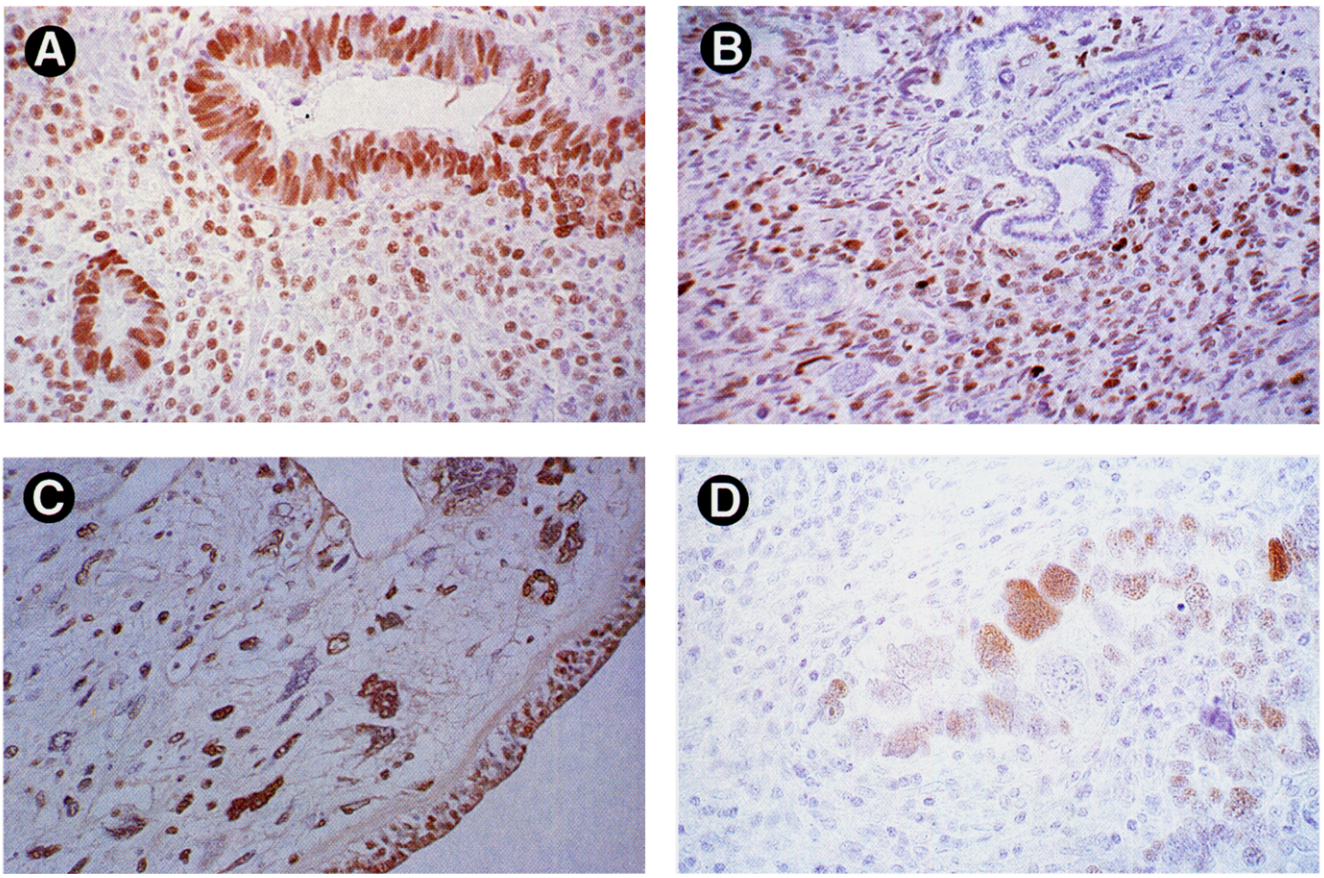

FIGURE 3. Immunohistochemical staining of pulmonary blastomas with mutations in the *p53* gene. (A) Both epithelial and stromal staining are prominent in case no. 4 (Table 2). (B) Most staining is stromal in case no. 5 (Table 2). (C) p53 protein is strongly expressed in pleomorphic nuclei from specimen two (Table 2). (D) Case no. 1 (Table 2) contained markedly pleomorphic nuclei that stained focally.

biphasic microscopic appearance, biphasic blastomas are clonal in their genetic makeup and protein expression. These immunohistochemical results show p53 protein staining in both epithelial and stromal regions of the BBs with identified *p53* gene mutations. In addition, the presence of a single dominant band in both SSCP and sequencing gels suggests that the *p53* gene mutations found represent the clonal expansion of a single mutated allele with loss of the wild-type allele.

In this study, the authors were not able to identify

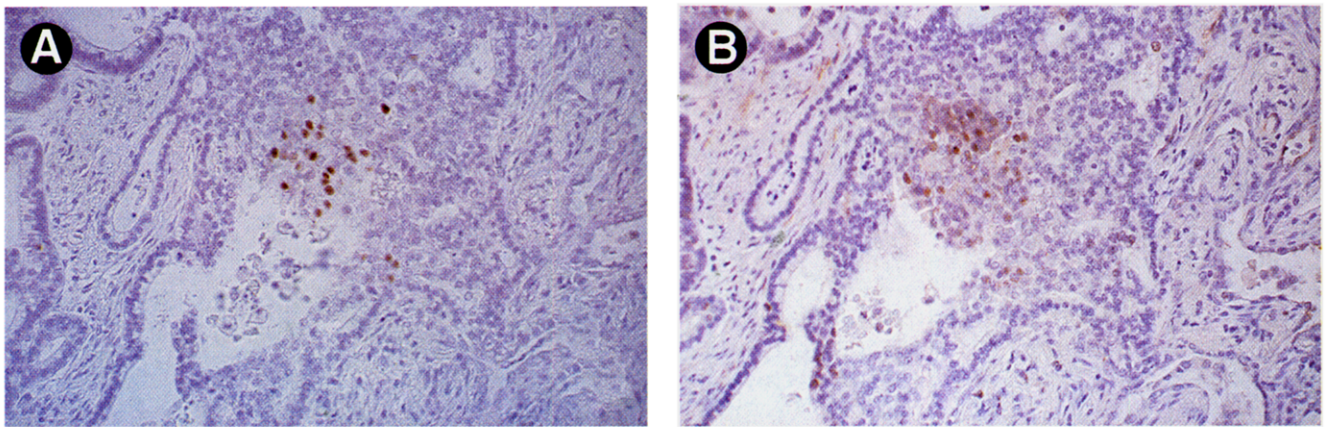

FIGURE 4. Immunohistochemical staining of a pulmonary blastoma without a mutation in the *p53* gene. (A) Staining for p53 protein with a D01/D07 cocktail shows scattered focal staining. (B) Staining for Ki67 in an immediately adjacent section shows scattered focal staining in the same region that stained similarly for p53.

p53 gene mutations or p53 protein overexpression in well-differentiated fetal adenocarcinomas. This may be consistent with their superior prognosis and may suggest a different biological basic of tumorgenicity in these lesions. Alternatively, it is possible that these mutations were present, but the authors did not find them. The detection of mutants by both SSCP and sequencing requires that a certain percentage of cells share a clonal abnormality. In the past, the authors have refined these techniques until they could identify relatively small tumor populations (8% to 25%)[25] mixed with normal tissue. In all cases of WDFA, although the histologically malignant epithelium was mixed with bland stroma, more than 40% of the cells were epithelial by light microscopy; therefore, the specimens were suitable for analysis with these techniques. Immunohistochemistry was also used because it allows simultaneous evaluation of both histology and protein expression; however, in no case of WDFA was more than 10% staining encountered.

All of the patients in this study with *p53* gene mutations and an available smoking history were smokers or had recently stopped smoking. Therefore, the authors evaluated the mutated sequences in the *p53* gene determined by sequencing. The transition mutations found in pulmonary blastomas, two at CpG dinucleotides, suggests that endogenous mutations may occur in this tumor. Endogenous mutations have been implicated in *p53* gene abnormalities in pediatric tumors and colon cancer.[26] One case of pulmonary blastoma showed a G:C → T:A mutation, which is related to tobacco smoke exposure and benzpyrene toxicity.[8] The patient with this mutation also had an extensive history of smoking supporting the theory that smoking might be implicated in the development of *p53* gene mutations. The other transversion that was identified was not as typical of a benzpyrene mutagenic effect because it occurred with the opposite strand orientation, inconsistent with the preferential repair mechanism of the transcribed strand.

The histological distinction between biphasic blastoma and WDFA has prognostic significance because the 10-year survival of BB is 20% and that of WDFA is 80%. In addition, the occurrence of *p53* gene mutations in biphasic blastoma suggests that molecular abnormalities may account for some of the differences between these tumors. However, in this small series, it is not possible to draw any conclusions regarding the independent significance of *p53* mutations on prognostic factors, such as survival, metastases, and stage of disease. It is possible that the frequency of mutations in the *p53* gene in pulmonary blastomas is higher than the findings in this study because only exons 5 to 9 were investigated by SSCP because of the small amount of tissue available. Mutations outside of exons 5 to 8 in the *p53* gene may also be missed by immunohistochemical screening because mutations in other exons and intronic regions do not produce observable p53 protein staining in other studies.[4,11,12]

In summary, mutations in the *p53* gene occur in pulmonary blastomas and these results show that they occur more frequently in biphasic blastomas than in well-differentiated fetal adenocarcinomas. These results provide the first indication of molecular abnormalities in these rare blastematous tumors and provide molecular support for the histological distinction of the tumor into these two subtypes. Mutations in the *p53* gene and protein overexpression occurred in both epithelial and stromal components of biphasic blastomas and support the concept that the tumor arises from a single clone that can differentiate into both stromal and epithelial morphologies. The types of *p53* gene mutations found (including mutations at CpG dinucleotides and a G → T transversion mutation) are similar to mutations found in other lung cancers and include a mutation associated with benzpyrene, a product of tobacco smoke. The presence of this benzpyrene mutation, as well as the history of smoking provided by all patients with pulmonary blastoma and *p53* gene mutations, suggests that smoking may be implicated in the development of some of these tumors. Immunohistochemistry and molecular analysis proved effective in analyzing limited quantities of paraffin-embedded, formalin-fixed clinical samples in this study.

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