Comparison of Immunofluorometry and Immunohistochemistry for the Detection of p53 Protein in Lung Cancer Specimens

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Although immunohistochemical techniques are widely used to demonstrate the presence of mutant p53 protein in a wide variety of malignant tissues, quantitative enzyme-linked immunosorbent assay (ELISA)–type immunoassays offer some advantages. In this study we compared immunohistochemistry, performed on formalin-fixed, paraffin-embedded sections of 91 primary lung tumor tissues, with a highly sensitive quantitative two-site immunofluorometric assay, on extracts of fresh-frozen specimens from adjacent regions of the same tissues. Monoclonal DO-7 antibody, and the related monoclonal DO-1 with polyclonal CM-1 antibodies, were used for immunostaining and ELISA, respectively. Concentrations of p53 were expressed relative to total protein, while an immunostaining score reflected the proportion of stained malignant cells, intensity of staining, and tumor cellularity. Strong concordance was shown between the two methods by Spearman correlation ($P<.001$), Wilcoxon rank sum ($P<.001$), and contingency table ($P<.001$) analyses. The use of ELISA-type assays for p53 quantification in lung tumor tissues may be an alternative to the more labor-intensive histologic techniques. (Key words: p53 protein; Enzyme-linked immunosorbent assay; Immunohistochemistry; Lung neoplasms) Am J Clin Pathol 1997;107:308–316.

Mutational inactivation of the p53 gene is the most frequent genetic event reported in the vast majority of human cancers.[1] Loss of p53 function, primarily by missense mutation of one p53 allele coupled with the deletion of the other, may result in the impairment of DNA repair,[2] cell cycle arrest,[3] or programmed cell death,[4] processes that together are believed to limit the propagation of mutations that cause malignancy. Wild-type p53 is a nuclear phosphoprotein that fulfills its role as a tumor suppressor by transactivation of target genes[5–7] or by inhibiting transcription from others,[8,9] possibly as a consequence of direct recognition of DNA damage.[10] Mutant p53 protein, in contrast, often has a dominant negative phenotype over its wild-type counterpart,[11] and because of its longer half-life, it accumulates within cells harboring a missense-mutated p53 gene.[12] Techniques to detect p53 gene mutation and protein overexpression have attracted tremendous clinical interest because many studies have suggested their utility for the early diagnosis of cancer,[13] for indication of unfavorable prognostic outcomes,[14–16] and for prediction of responsiveness to antineoplastic therapies.[17]

The strong correlation between p53 gene mutation and p53 protein accumulation existing under most but not all circumstances,[18] permits simple immunologic methods for p53 protein detection to substitute for the more complex and labor-intensive procedures for determining p53 alterations at the nucleic acid level. Most commonly, these immunologic methods take the form of well-established immunohistochemical techniques that are rapid, technically nondemanding, and able to provide antigen localization for the histologic features of the tumor. However, methodologic differences resulting from the various combinations of tissue fixation and antigen unmasking procedures,[19–22] primary anti-p53 antibody (performance of which may be

affected by specimen processing),[19-21] and criteria for designating the p53 expression status of specimens[22] have made cross-study comparisons of immunohistochemically detected p53 protein difficult. An alternative to immunohistochemistry (IHC) is the measurement of soluble p53 protein in tissue extracts by quantitative immunoassay. Although such enzyme-linked immunosorbent assay (ELISA)–type methods likewise suffer from inherent pitfalls, including the requirement for fresh-frozen tissue and the inability to localize p53 expression to cellular or tissue components, they may offer several advantages.[23] Most noteworthy are that they may be performed with minimal technical expertise, they generate numerical results amenable to objective and consistent interpretation, and they provide enhanced specificity owing to the use of two p53-specific antibodies (the most common assay configuration).

Relative to the widespread use of IHC for the demonstration of p53 protein overexpression in human tumor tissues, ELISAs of p53 protein have been used much less frequently.[24-26] One of these ELISA methods, among the first developed,[25] has been used to determine p53 protein concentrations in extracts from tumors of breast,[27,28] gastrointestinal,[27,29] and vulval origin[27] and has been shown to be comparable to IHC, when performed in parallel, for the assessment of p53 protein status in breast[28] and gastrointestinal[29] tumor specimens. Recently, we described a highly sensitive ELISA of p53 protein,[30] incorporating a time-resolved fluorescence detection system. Because p53 protein concentrations in primary lung tumor extracts have not yet been compared to the degrees of immunostaining performed on the same specimens, we conducted this study to determine whether the levels of p53 expression in lung tumor tissue demonstrated by our immunofluorometric assay were concordant with results obtained by a conventional immunohistochemical technique.

MATERIALS AND METHODS

Tumor Specimens

This study received the approval of the ethics and research committee at St Joseph's Health Centre, Toronto, Ontario, Canada. Surgically resected lung tumor tissues were obtained from 91 patients operated on at St Joseph's Health Centre between June 9, 1993, and March 22, 1995, for the treatment of primary lung carcinoma. Table 1 gives the distributions of patients by age at operation, TNM stage,[31] and histologic grade and type according to the World Health Organization criteria.[32] Immediately following the operation, representative tissue samples of each lung tumor were

selected and partitioned into two equivalent portions. While one portion was fixed in 10% neutral buffered formalin and embedded in a permanent paraffin block, the other was snap frozen on dry ice and stored at -80°C for no more than 6 months until the extraction and ELISA procedures were performed.

Soluble Extracts

Frozen lung tumor tissues (~0.2 g) were pulverized on dry ice to a fine powder, which was suspended in 1 mL lysis buffer (50 mmol/L tromethamine [tris], 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mL/L NP-40 surfactant, 10 mg/L phenylmethylsulfonylfluoride, and 1 mg/L each of aprotinin and leupeptin) and incubated for 30 minutes on ice before centrifugation at 14,000g for 30 minutes at 4°C to collect the supernatants. The crude cell lysates were immediately assayed for p53 protein by using immunofluorometry and for total protein content by using a kit based on the bicinchoninic acid method (Pierce Chemical, Rockford, Ill). Ten tumor tissues were further sampled by cutting, from each of three different surfaces, approximately 0.2-g portions of tissue that were independently pulverized and extracted as described. Extracts from histologically normal lung tissue, cut from the resection margins of seven lung tumors, served as negative tissue controls.

Immunofluorometric Assay

A "sandwich-type" ELISA, briefly outlined here and described in detail elsewhere,[30] was used to

TABLE 1. CHARACTERISTICS OF PATIENT POPULATION

Age, y	N	Stage*	N	Grade†	N
<50	10	I	54	G1	6
51–60	15	II	16	G2	54
61–70	37	IIIA	12	G3	26
71–80	24	IIIB	1	G4	1
>80	5	IV	4		

Histologic Type	N
Adenocarcinoma	43
Squamous cell carcinoma	36
Carcinoid tumor	4
Small cell carcinoma	3
Large cell carcinoma	2
Adenosquamous carcinoma	1
Adenoid cyslic carcinoma	1
Carcinosarcoma	1

*Stage unknown for 4 patients.
†Grade unknown for 4 patients.

measure the p53 protein concentrations in the lung tumor extracts. Soluble p53 protein, present in extracts and calibrators diluted twofold in buffer A (50 mmol/L tris at pH 7.80, 60 g/L bovine serum albumin, and 0.5 g/L NaN_3) supplemented with 0.5 mol/L KCl, 10 mL/L mouse serum, and 5 mL/L polysorbate 20 (Tween 20) detergent, was first immobilized in microtiter wells coated with monoclonal DO-1 antibody (gift of Dr David Lane, University of Dundee, Dundee, Scotland), which recognizes the same epitope on the surface of p53 protein as the related DO-7 antibody[25] used for the immunohistochemical staining procedure. Following the initial 3-hour incubation at 37°C, bound p53 protein was detected by subsequent 1-hour incubations at room temperature with polyclonal CM-1 antiserum (Novocastra, Newcastle upon Tyne, England) raised in a rabbit host against recombinant wild-type human p53 and diluted 5,000-fold in buffer A, and then with alkaline phosphatase–conjugated goat antirabbit immunoglobulin diluted to 120 µg/L in buffer A containing 0.5 mol/L KCl and 100 mL/L goat serum. Hydrolysis of the enzyme substrate (0.01 mol/L diflunisal phosphate in 0.1 mol/L NaOH, diluted 10-fold in 0.1 mol/L tris at pH 9.10, containing 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$ and 0.5 g/L NaN_3), added for 10 minutes at room temperature, yielded a product that entered into a fluorescent complex when the developing solution (1 mol/L tris, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA) was also added. Fluorescence at 615 nm was measured after 1 minute by a Cyberfluor-615 Immunoanalyzer (Cyberfluor, Toronto, Ontario, Canada) in a time-resolved mode, which greatly reduces the background fluorescence signal.[33] All reagents were added to wells in 100-µL volumes. Concentrations of p53 were interpolated from a calibration curve generated by the simultaneous assay of a dilution series of an extract of Sf9 insect cells infected with a p53-expressing baculovirus vector (gift of Dr Thierry Soussi, INSERM, Institut de Génétique Moléculaire, France), as described previously.[30] Values of these calibrators, ranging from 0 to 75 µg/L, were established based on the assay of reconstituted preparations of premeasured, lyophilized recombinant human p53 protein (Oncogene Science, Uniondale, NY). Analytical characteristics of the ELISA include a sensitivity of ~0.04 µg/L and a linear response range from 0.15 to 75 µg/L. Concentrations of p53 protein in the lung tumor extracts were expressed relative to the total protein content. Because the epitope recognized by DO-1 antibody is within an amino terminal domain shared by all conformations of p53 protein,[25] the ELISA is able to detect mutant and wild-type p53 protein.

Immunohistochemistry

Sections (4–5 µm) of the same paraffin-embedded tissues used to make the histologic diagnosis in each case were placed on silanated slides, dried overnight at 65°C, dewaxed and rehydrated in xylene and graded concentrations of alcohol to distilled water, and treated with methanol hydrogen peroxide. Microwave antigen retrieval[34] was performed in a 1.75-kW microwave oven (Litton-Moffat, Rexdale, Ontario, Canada) at the high setting for 30 minutes in a 10 mmol/L citrate buffer, pH 6.0, after which the slides were incubated for 30 minutes in the same buffer before being transferred into phosphate-buffered saline (PBS; 150 mmol/L NaCl, 10 mmol/L Na_2HPO_4, 2 mmol/L $NaKH_2PO_4$, pH 7.2). The slides were then incubated for 5 minutes with 2% nonimmune goat serum in PBS before applying monoclonal DO-7 antibody (Novocastra), diluted 50-fold in Dako Diluent Buffer (Dako, Glostrup, Denmark), for a 1.5-hour incubation. After washing the slides twice in PBS, bound complexes were detected by a subsequent 45-minute incubation with biotinylated goat antimouse antibody (Dako) diluted 200-fold in PBS by washing twice as before and by a 45-minute incubation with horseradish peroxidase–conjugated streptavidin (Dako) diluted 300-fold in PBS. All the aforementioned incubations were performed at 37°C in a humidified chamber. Washing of the slides again two times was followed by incubation for 4 minutes at room temperature with the chromogen, a solution of 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/mL) and 0.009% hydrogen peroxide in tris-buffered saline (150 mmol/L NaCl, 50 mmol/L tris, pH 7.6). The slides were then lightly counterstained with Harris' hematoxylin and immersed in acid alcohol, graded alcohols, and xylene, and finally coverslipped. Tissues derived from an astrocytoma, an ovarian carcinoma, and a ductal breast carcinoma, all previously shown to exhibit strong staining for p53 protein using DO-7, were sectioned and processed, as described herein, in parallel as positive controls. Negative controls for each lung tumor specimen were provided by omitting the DO-7 antibody.

Scoring System

A simple scoring scheme, similar to that used by another group,[28,29] was used to semiquantify the degree of immunohistochemical staining of the lung tumor sections. Scoring was performed by examination of each DO-7 antibody–stained section by a single

pathologist who was unaware of the corresponding p53 ELISA result. Three parameters were used for scoring, namely the proportion of malignant cells stained, the intensity of staining, and the tumor cellularity. To account for differences in tumor cellularity among specimens, a value of 1 (low), 2 (intermediate), or 3 (high) was assigned to the corresponding hematoxylin-eosin–stained section, viewed under scanning power (×4), to categorize it for the amount of neoplastic tissue present (eg, excluding regions of necrosis or stroma). To reflect the proportion of malignant cells stained in each section, 10 nonoverlapping, randomly chosen low power (×10) fields were each given an integer value of 1, 2, 3, or 4 corresponding approximately to 0% to 25%, 26% to 50%, 51% to 75%, or 76% to 100% staining. Ten additional higher power (×20) fields chosen in a similar manner were also each given a value from 0 (absent) to 3 (dark) to represent the intensity of staining within the malignant cells in each section. Summation of the mean of the 10 values reflecting the proportion of stained cells and the mean of the 10 values reflecting the staining intensity yielded the raw score (range, 1–7) in each case. Finally, the overall score (range, 1–21) was calculated by multiplication of the raw score and the cellularity value in each case.

Statistical Analysis

The relationship between the concentrations of p53 protein determined by ELISA and the corresponding cellularity-adjusted IHC scores was examined by Spearman correlation analysis, Wilcoxon rank sum tests, and contingency table analysis, all performed using SAS version 6.02 software (SAS Institute, Cary, NC). For the latter two procedures, tumor specimens were classified as p53 negative or p53 positive by each of the detection methods under comparison based on cutoff points for p53-positivity equal to the median protein-adjusted p53 concentration in the case of the ELISA results and equal to the median cellularity-adjusted staining score in the case of the IHC findings.

RESULTS

In all 91 extracts prepared from the frozen lung tumor tissues, the p53 protein concentrations exceeded the detection limit of the ELISA; the distribution of values ranged from 0.04 µg/L to 70.69 µg/L, with a median, mean, and standard deviation of 0.55 µg/L, 6.03 µg/L, and 12.81 µg/L, respectively. When the p53 protein concentrations were divided by the amount of protein present, the distribution of values
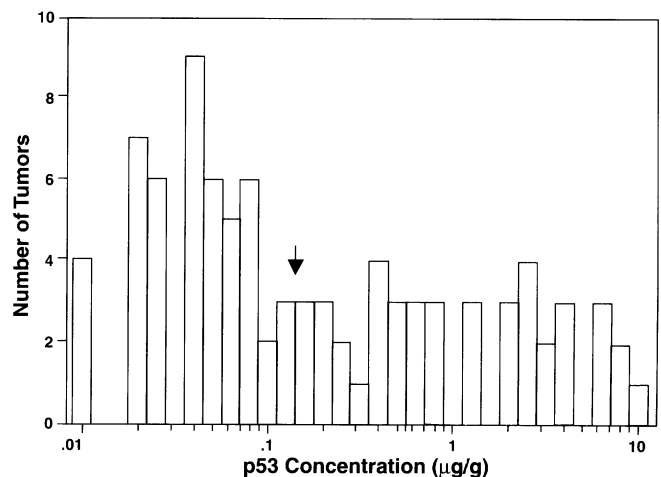

Fig 1. Distribution of p53 protein concentrations in lung tumor extracts measured by immunofluorometric assay. Values are expressed relative to the total protein content of the extracts. The arrow indicates the median value used as the cutoff point for p53-positivity.

expressed as µg/g protein (Fig 1), had the following characteristics: minimum, 0.01 µg/g; maximum, 10.97 µg/g; median, 0.13 µg/g; mean, 1.13 µg/g; and standard deviation, 2.15 µg/g. Evidence that these tumor tissues displayed minimal heterogeneity in p53 protein overexpression was provided by further sampling of a subset of tumors (n = 10) in which protein-adjusted p53 concentrations, determined in three different samples of tissue removed from nonadjacent surfaces and then independently extracted and assayed for p53 and for total protein, were revealed to vary by no more than 10% for each specimen (data not shown). For statistical analysis, the median protein-adjusted p53 concentration of 0.13 µg/g was arbitrarily selected as the cutoff point at which, and beyond, p53 concentrations were considered positive. Compared with these extracts of neoplastic tissues, those of a small number (n = 7) of normal tissues bordering regions of overt malignancy differed markedly in p53 protein content; p53 concentrations in these control specimens were no greater than 0.07 µg/g.

Light microscopic examination of the matched formalin-fixed, paraffin-embedded sections on which immunohistochemical staining was performed revealed predominantly nuclear staining, except in occasional cases of squamous cell carcinoma in which only faint cytoplasmic staining was observed. These latter cases were considered p53 negative by IHC. In all cases in which immunostaining was present, however, it was confined to malignant cells; normal

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epithelia and stroma were consistently negative for immunostaining. Although some sections that were positive for staining displayed focal clustering of stained tumor cells when viewed under low-power (×10) magnification, in others the staining was more diffuse throughout the specimen. No obvious relationship was observed among the number of cells staining, the intensity of staining, and the tumor cellularity in these sections. For comparison, the p53 immunostaining of the astrocytoma, breast carcinoma, and ovarian carcinoma sections used as positive controls were moderately intense. In contrast, immunostaining was completely absent for every specimen control in which the primary antibody was omitted.

Conversion of these visual findings into numerical scores composed of three categorical variables, tumor cellularity, frequency of malignant cell staining, and intensity of malignant cell staining, allowed the integration of these qualitative features into single values representative of the overall extent of staining of the sections. Like the distribution of p53 protein concentrations measured by the ELISA, that of the immunostaining scores (Fig 2), which ranged from 1 to 20.7, was also skewed positively. Specimens with scores equal to or greater than the median value of 3.6 were arbitrarily denoted as p53 positive by IHC. Figure 3 shows DO-7-stained sections representative of cases in which weak staining occurs in a small proportion of malignant cells or in which a high proportion of the tumor cells are intensely stained. Because 10 separate

Fig 3. Immunohistochemical staining for p53 protein using monoclonal DO-7 antibody showing A, fewer cells (<25%) with low intensity staining (score = 1) and B, many cells (>75%) with high intensity staining (score = 3) (original magnification ×500).
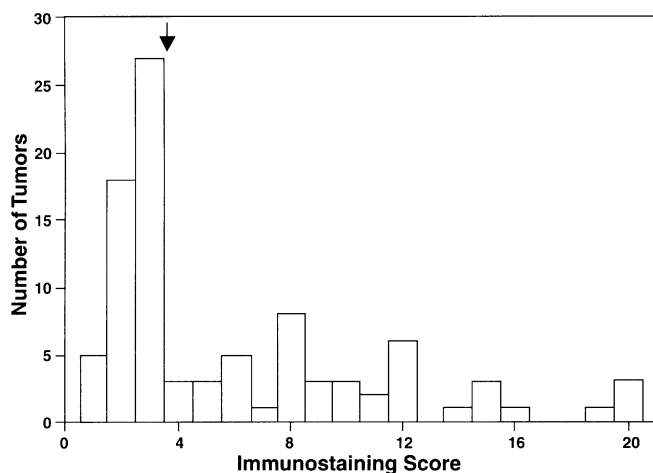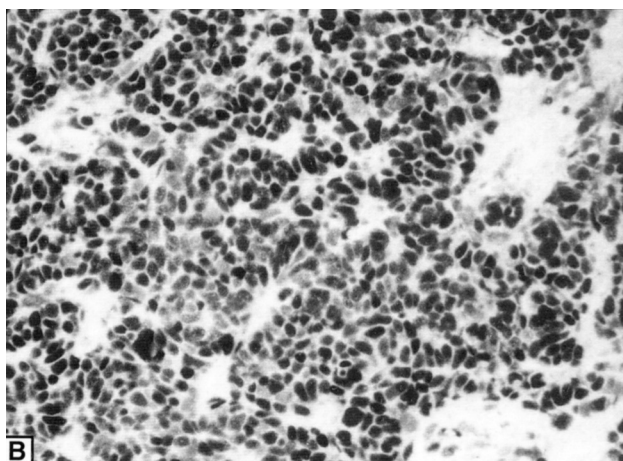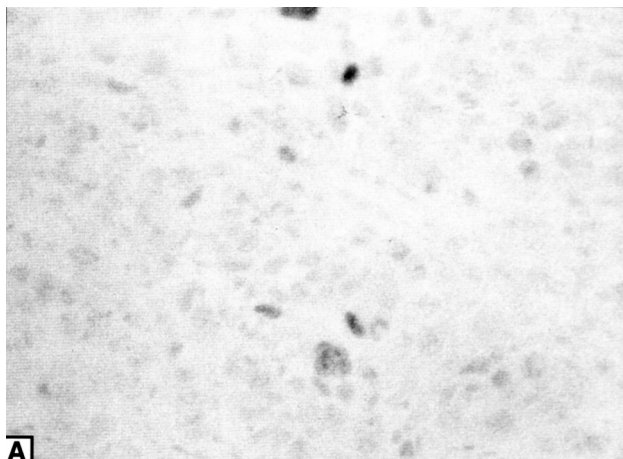

Fig 2. Distribution of immunostaining scores using the anti-p53 monoclonal antibody DO-7 on formalin-fixed, paraffin-embedded lung tumor tissues treated for antigen retrieval by microwave heating. The arrow indicates the median score used as the cutoff point for p53-positivity. See the "Materials and Methods" section for details.

microscopic fields of each section were examined to evaluate the number of stained tumor cells, and 10 other fields were used to judge the intensity of staining, estimates of the variability within given specimens for these characteristics could also be made. The majority of sections displayed DO-7 staining patterns that were homogeneous for the proportion of malignant cells taking up stain and the intensity of such staining (data not shown). While only a few (n = 10) sections were in the low tumor cellularity group, most had tumor cellularities that were intermediate (n = 38) or high (n = 43). No statistical relationship could be found between the cellularity values and the corresponding mean proportions of staining cells or mean intensities of staining (data not shown). However, sections in which a large number of tumor cells stained

for p53 protein also tended to be those that had intense immunostaining, indicated by the strong correlation ($r = .91$; $P = .01$) between the mean values reflecting these two parameters.

The degree of concordance between the results of the two techniques for p53 protein detection was first examined by Spearman correlation analysis, by which the methods were shown to be positively correlated ($r = .65$; $P<.001$). The Wilcoxon rank sum test was also applied in two formats. In the first, specimens divided into two groups on the basis of p53 protein concentrations, determined by ELISA, which were below ($n = 46$) or above ($n = 45$) the median p53 protein level of 0.13 µg/g were revealed to differ significantly ($P<.001$) in their matched IHC scores. Specifically, the group of tumor specimens negative for the p53 protein by ELISA had a lower median staining score (equal to 3; range 1–8.4) than the median score (equal to 9.3; range 2–20.7) found in specimens in which the p53 protein concentrations were greater than 0.13 µg/g. In the second configuration of the Wilcoxon rank sum test, an IHC score of 3.6 was used as the cutoff point to dichotomize specimens into p53-negative ($n = 43$) and p53-positive ($n = 48$) groups, for which respective distributions of p53 protein concentrations were compared. The p53 protein levels in specimens categorized as p53 negative by immunostaining were thus shown to be significantly lower (median, 0.06 µg/g; range, 0.01–0.76 µg/g) than the p53 concentrations detected in the p53-positive group (median, 0.75; range, 0.01–10.97; $P<.001$). Finally, when matched tissue extracts and paraffin sections were simultaneously classified as either p53 negative or p53 positive by both ELISA and IHC, contingency table analysis (Table 2) showed a significant association ($\chi^2=35.9$; $P<.001$) between the two systems of classifying tumors for p53 protein status. As shown in Table 2, 83.7% of lung tumor specimens in which the DO-7–stained sections had scores less than 3.6 yielded extracts in which p53 protein levels were below the cutoff point for p53 positivity by ELISA. Similar agreement was demonstrated by specimens classified by IHC as p53 positive, of which 79.2% had matched frozen tissues which had p53 protein concentrations greater than 0.13 µg/g. Also shown in Table 2 are the 17 cases in which IHC and ELISA yielded contradictory findings. Neither the seven IHC-negative, ELISA-positive cases (in which p53 protein concentrations were no greater than 0.76 µg/g) nor the 10 IHC-positive, ELISA-negative cases (in which immunostaining scores ranged from 3.6 to 8.4) could be explained by clustering within a given

stage, grade, or histotype (data not shown). Reexamination of the immunostained sections of these discrepant cases confirmed the earlier scoring results. However, three of the 10 cases that were p53 negative by ELISA were only borderline positive by IHC, having immunostaining scores equal to the median value of 3.6, used as the cutoff. Of the seven cases in which positive ELISA findings were accompanied by negative IHC results, one case had a p53 protein concentration equal to the cutoff point of 0.13 µg/g, and two cases had p53 protein levels of 0.14 µg/g, just beyond the cutoff point. Another of the same seven cases had an immunostaining score of 3.4, just below the median value. Five of the 10 ELISA-negative cases were also revealed to have achieved positive overall IHC scores by the multiplication of raw scores, indicating sparse staining of weak to moderate intensity, by high tumor cellularity values. To ensure homogeneity in the sampling of frozen tissues for extraction, 10 of the 17 discordant specimens were sampled at three different sites, re-extracted, and assayed for p53 protein. The variability among the three samples taken from each of the frozen specimens did not exceed 10%. These results clearly show that the parallel assessments of p53 expression status by ELISA and IHC yielded concordant findings in matched lung cancer specimens, all but three of which were nonsmall-cell lung carcinomas. The cell extracts prepared from the small-cell lung carcinomas had p53 protein concentrations of 0.02 µg/g, 1.49 µg/g, and 33.48 µg/g that agreed with the immunostaining scores of 3, 10.4, and 20.4, respectively, determined in the matched paraffin sections.

DISCUSSION

Lung cancer is the leading cause of cancer-related deaths in Western countries.[35] Mutation of the p53

TABLE 2. CONTINGENCY TABLE ANALYSIS OF P53 PROTEIN STATUS DETERMINED BY IMMUNOFLUOROMETRY (ELISA) AND IMMUNOHISTOCHEMISTRY (IHC)

	p53 Protein Status by ELISA* Number of Patients (%)		
	Negative	Positive	P Value
p53 protein status by IHC[†]			
Negative score	36 (83.7)	7 (16.3)	
Positive score	10 (20.8)	38 (79.2)	<0.001

*ELISA=enzyme-linked immunosorbent assay. Cutoff for ELISA: negative <0.13 µg/g; positive ≥0.13 µg/g.

[†]Cutoff for IHC: negative <3.6, positive, ≥3.6. See text for scoring details.

gene and loss of heterozygosity on chromosome 17p13.3 occur in up to 75% of lung tumors.[36] Genetic abnormalities at the p53 gene locus are usually accompanied by the expression of stabilized mutant p53 protein,[37] detected in 30% to 70% of resected lung cancers[16,38-40] and demonstrated to correlate with clinicopathologic factors associated with poor prognosis[37,40] and with resistance to chemotherapy[41] and to be an independent predictor of reduced overall survival.[16,42] However, some investigators have failed to find prognostic value in p53 protein overexpression[37,38] or have shown that p53 protein may, in fact, predict a favorable clinical outcome in subsets of patients with lung cancer.[39,43] An association between survival of patients with lung cancer and p53 protein levels in tumor tissue might be obscured by the lack of an absolute correlation between intracellular accumulation of p53 and its functional status,[18] which might have a direct influence on tumor aggressiveness. The different conclusions relating to the prognostic utility of p53 protein in lung cancer could be also due to differences in patient populations, especially the histologic types represented, or to the analytical methods used to assess the p53 expression level of the tumor tissues. For the most part, these detection methods have been standard immunohistochemical procedures.

IHC is a rapid and simple technique, routinely practiced in most histopathology laboratories. Unlike biochemical analysis, it can identify distinct staining patterns at single cell resolution that could be clinically relevant.[18] The major disadvantage often cited of IHC is the significant rate of false-negative and false-positive predictions of the mutational state of the p53 gene.[44] The choice of fixative[19,22,44] may affect the staining intensity and distribution of stained cells using a single anti-p53 antibody. The section pretreatment, such as by enzymatic digestion or by microwaving to unmask otherwise cryptic p53 epitopes,[20,21,44,45] is another example. Because monoclonal anti-p53 antibodies differ in their epitope specificities and p53 mutants may vary in terms of epitope expression, selection of the primary immunoreagent is probably the single most important factor determining the success of IHC for p53 protein detection. It was therefore suggested that a cocktail consisting of at least three antibodies, one recognizing each of the three functional domains of p53 protein, might enhance detection sensitivity.[45] Monoclonal antibodies are also differentially sensitive to fixation-induced epitope loss. As a consequence, certain antibodies may simply be ineffective on sections processed in particular fixatives,[19,25] and although antigen retrieval

procedures have solved this problem in many cases, these techniques may also differ in effectiveness depending on the antibody used for p53 detection.[21,45,46] In addition, storage of paraffin slides for prolonged periods before staining has been associated with loss of p53 immunoreactivity.[47] Finally, there is the subjective nature of interpreting IHC, image analysis being the exception, and the subdivision of specimens (often simply into p53-negative and p53-positive groups) based on arbitrary criteria. Numerous scoring systems for designating the p53 status of tumor tissues have been devised that differ widely in complexity. Such lack of consensus as to what constitutes positive p53 expression, together with the wide range of immunohistochemical procedures used in the different studies, has meant that the results of p53-immunostaining in any tissue must be interpreted cautiously.[44]

Although ELISA-type immunochemical assays have been applied mostly to serum, they have also been used for other fluid matrices, including cell extracts from pulverized tissues. All ELISAs of p53 protein developed to date,[24-26,30] including the two that are commercially available (Oncogene Science), are of a "sandwich" configuration in which soluble p53 is immobilized between a solid phase monoclonal antibody recognizing mutant, wild-type, or both forms of p53, and enzyme-labeled polyclonal antibodies. Because the signal intensities are quantitative, ELISAs are far less subjective and obviate the high level of professional training needed to meaningfully interpret the results of immunostaining in a standardized manner. Furthermore, the requirement for p53 protein to simultaneously bind two immunoreagents and the rigorous multiple washing steps between incubations may impart to ELISAs a greater degree of specificity. Greater sensitivity may also result from the reduced background signal in the tumor extracts, due to washing steps and the use of a sample matrix containing only soluble components. Antigen unmasking is therefore not required in ELISAs. However, the major limitation of ELISAs is the requirement for fresh frozen tissue, because they cannot be applied to fixed tissue. Another major disadvantage of ELISAs for p53 is related to specimen processing. Pulverization of the tissue, necessary for evenly distributed cell lysis, destroys all tissue architecture and, hence, any information about the relationship between p53 expression and histologic features. The p53 concentration in each extract simply represents the average p53 protein level throughout the portion of the tissue sampled.

Comparison between ELISA and IHC for p53 protein detection, performed in parallel on the same tumor tissues, has already been reported for breast,[28] colon,[29] and gastric[29] cancers. These authors found statistically significant correlations between p53 protein concentrations in frozen tissue, measured by an ELISA method using DO-1 and CM-1 antibodies, and the p53 immunostaining scores using polyclonal CM-1 antiserum in sections of matched formalin-fixed, paraffin-embedded tissues using a scoring system that we used in this study. However, because there were cases in both studies in which ELISA failed to confirm the p53-positive status indicated by IHC, the latter technique was claimed to be more sensitive.

Several studies have demonstrated the utility of monoclonal DO-7 as the primary antibody for p53-immunostaining of formalin-fixed, paraffin-embedded lung carcinomas,[42–45] especially in conjunction with epitope retrieval techniques.[45,46] Because DO-7 recognizes the same linear sequence of amino acids as does DO-1, and both were shown to be equally suited for immunohistochemical detection of p53 in breast carcinomas, they may be considered equivalent in their affinities for p53 protein.[25] However, the immunoreactivity of DO-1 has been shown to be unaffected by microwaving,[46] whereas staining with DO-7 is enhanced by microwave heating[20,21,45,46] but is impaired by enzymatic digestion of the specimens.[21,46]

Our finding that p53-immunostaining scores correlated with p53 protein concentrations measured by ELISA, confirmed by Wilcoxon and contingency table analyses, was consistent with the concordance between these methods for detecting p53 protein accumulation reported earlier for breast[28] and gastrointestinal[29] tumors. In these other studies, however, each method resulted in the classification of a small number of specimens for p53 protein status that were discordant. While neither IHC-negative, ELISA-positive nor IHC-positive, ELISA-negative cases could be explained on the basis of any particular clinicopathologic or histologic feature, some of the discrepant cases could be explained by an artifact related to the selection of cutoff points, with either of the methods giving results just below or above the cutoff point used to dichotomize specimens as p53 negative or p53 positive. Sampling variation could not explain the ELISA results, although this factor could not be ruled out for IHC because only one sampling site was used. In the remaining cases, other factors relating to specimen handling before p53 analyses may have played a part. For example, partitioning of each lung tumor specimen in the operating room into portions that were not equivalent for the

amount of p53-expressing cells could have led to discrepant findings between the methods. Degradation of p53 protein by autolysis of fresh tissue not immediately frozen could have led to false-negative results by ELISA despite efforts to freeze each specimen promptly. Conversely, false-negative findings by IHC might have occurred because of failure of microwave irradiation to unmask p53 protein, perhaps as a consequence of overfixation. Because controls to determine the effectiveness of antigen retrieval were not performed, we could not address this latter possibility. For determining p53 protein expression status, no "gold standard" method exists against which the results of IHC and ELISA could have been compared. However, techniques to reveal the mutational status of the p53 gene or to detect p53 messenger RNA transcripts would have provided valuable correlates to the two methods compared in this study, but they were not performed. Nevertheless, this study indicates that sensitive immunofluorometry for p53 protein in lung tumor tissues gives results that are highly concordant with those obtained by standard IHC and, therefore, is an alternative approach for the determination of p53 overexpression in malignant lung tissue.

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