Clinical Utility of the Immunocytochemical Detection of p53 Protein in Cytological Specimens

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ABSTRACT

In the important cytopathological distinction between benign and malignant lesions, there is always a residue of suspicious cases which cannot be satisfactorily diagnosed. Overexpression of the p53 tumor suppressor gene product has been consistently correlated with p53 missense gene mutation and is associated with malignancy. Therefore, assessment of p53 expression may assist in the cytopathological diagnosis of malignancy. Immunohistochemical assessment of p53 expression has been performed on a prospective series of 1333 non-gynecological cytological specimens in the setting of a teaching hospital group. Evaluation of p53 staining was performed without knowledge of cytopathological diagnosis. Resultant p53 expression data were correlated with cytopathological diagnosis and clinical information. Of the 999 assessable cases, 956 had a clear cytopathological diagnosis. In these, p53 overexpression occurred in 108 cases of which 86 were malignant lesions. Of the 848 p53-negative cases, 119 were in fact neoplasms. The false positives were predominantly (19 of 22) reactive mesothelial proliferations, and overexpression occurred in only a small proportion of cells. While the sensitivity of p53 overexpression is low (p53 overexpression only occurring in 41.9% of tumors), the overall specificity is 97%. In the 43 cytopathologically suspicious cases, 7 were p53 positive, all of which proved to be malignant. In this prospective, unselected series, we have conclusively demonstrated a close correlation between overexpression of p53 protein and neoplasia. Furthermore, we have shown the possible utility of p53 immunostaining in cytopathology. While there are important technical caveats and some cytological specimens are suboptimal for immunostaining, the data indicate that assessment of p53 is a valuable adjunct to morphological assessment in the analysis of cytologically suspicious cases.

INTRODUCTION

The advantages of cytological analysis of FNAs and the analysis of cells from ascitic, pleural, and other fluids include rapidity of diagnosis, minimal morbidity, and reduced expense. However, such methods have the disadvantage of requiring considerable skill in interpretation of cytological preparations. While the confident diagnosis, whether benign or malignant, is possible in the majority of cases, there remains a significant proportion of cases in clinical practice in which the findings can only be reported as suspicious (1). Consequently, the availability of techniques that would enhance the diagnostic accuracy and robustness of cytological methods and reduce the residue of uncertain diagnoses would be of considerable clinical value.

Application of our burgeoning understanding of the molecular basis may prove to be of value in diagnostic cytopathology, and one attractive area for study is the p53 tumor suppressor gene and its product. The currently favored model of p53 function proposes that the product of the p53 gene has an important role in the sensing of, or response to, DNA damage, acting as a potent transcriptional regulator. Induction of p53 expression is associated with profound growth arrest, presumably allowing repair of damaged DNA (2–6). Involvement in apoptosis, possibly as a means of altruistic suicide of severely damaged cells, can also occur (7–9). A considerable body of evidence points to p53 being a tumor suppressor gene, and abnormalities of this gene have been found to be the most commonly occurring single genetic lesion occurring in a wide variety of tumors (10–14). The function of p53 can be lost from the cell by a number of mechanisms potentially leading to promotion of a tumorigenic phenotype (15, 16). The p53 protein may be sequestered by cellular and viral oncogene products such as mdm2 (17, 18) and E6 ORF of human papilloma virus types 16 and 18, respectively (19). Alternatively, somatic missense mutations, particularly in highly conserved regions of the gene, may occur (14, 15). Mutation of only one allele is sufficient to cause loss of the p53 function by a dominant negative effect since mutant protein oligomerizes with the remaining wild-type protein (20). Allelic deletion of the remaining wild-type gene often follows (21), and functional inactivation of p53 is associated with progressive genetic instability (22–24).

It has been consistently found that mutant p53 protein, which takes on an abnormal conformation, is much more stable than wild-type protein, accumulates in neoplastic cells (14, 25, 26), and, thus, becomes immunologically detectable. Therefore, positive immunostaining is indicative of abnormalities of the p53 gene or its product (26, 27). However it is notable that immunohistochemical detection of p53 has been reported in cells under certain circumstances such as following genotoxic insult (2–5). With this caveat, given that p53 mutations occur frequently in malignant cells and the consequent elevation in p53 expression can be conveniently detected by immunocytochemical methods, there is a potential role for p53 staining in cytopathology as a discriminant of neoplastic cells. A small pilot study provided support for this proposition in cytological material (28). Therefore, we sought to test the value of p53 staining in a large prospective series of cases.

MATERIALS AND METHODS

Case Material. Cytological specimens from all sites (with the specific exception of exfoliative cervical cytology and mucosal brushes from other sites received already fixed and smeared onto slides) were prospectively accrued from the cytopathology departments of the three hospitals in our group: Guy's, St. Thomas's, and Lewisham. Clinical samples included urine, sputa, bronchial washings, ovarian cyst fluids, pleural and peritoneal aspirates, FNA samples from visceral sites, and tissue imprints. The only criterion for exclusion was insufficient spare material after conventional preparations had been made. During a 1-year period 1333 cases were accrued, but 334 were subsequently excluded due to inadequacy of the sample (n = 229), uninterpretable immunostaining result (n = 53), and assorted technical problems (n = 52). This left 999 assessable cases.

Immunohistochemistry. Specimens were prepared in the routine manner on uncoated slides, air dried, and subsequently fixed. This left 999 assessable cases. 

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3 The abbreviation used is: FNA, fine-needle aspirate.
enous peroxidase with 1% H$_2$O$_2$ in Tris-buffered saline (pH 7.4) for 20 min. After blockade of nonspecific binding sites using 5% solution of normal rabbit serum, immunological detection of p53 was performed using the Do-1 murine monoclonal antibody (30). Antibody was used at 1:100 dilution with incubations at 4°C overnight. Primary antibody was detected using an indirect immunoperoxidase system (Dakopatts UK PLC) and diaminobenzidine as a substrate. A light hematoxylin counterstain was then applied, followed by dehydration, clearing, and mounting. In some examples of reactive mesothelial proliferation, replicate samples were stained using the polyclonal anti-p53 serum CM-1 (31).

Conventional immunocytochemical controls were used, including omission of primary antibody and the use of an irrelevant antibody as a control for the detection system. Cytosplasms of PANC-1 cells (American Type Culture Collection) were used as positive controls (known to overexpress p53 as a consequence of a mutation CGT273 to CAT273; arginine to histidine), and cytosplasms of human diploid fibroblasts were used as negative controls (32, 33). For cases in which relevant histological material was available, immunohistochemistry was performed on paraffin sections using the ABC method as recommended by the supplier (Dakopatts).

Assessment. Cytopathological diagnosis was made using conventional criteria by N. W. D. or P. O. G. W. Entirely independent of this, immunostained cytological preparations were assessed by S. P. D. This involved screening of the entire slide and assessing the number and nature of p53-labeled cells. Only clear nuclear staining was accepted as positive. Equivocal cases were assessed by a second observer (P. A. H.). Staining considered equivocal by both observers on immunocytology were then deemed negative. After this, correlation of the independently determined cytological diagnosis and p53 status was performed using contingency tables with determination of sensitivity, specificity, accuracy, and predictive values. Cases for which a cytopathological diagnosis of malignancy was made but for which no p53 expression was detectable were further reviewed. Where available, relevant histological material was immunostained for p53 from specimens in all of the categories except true negatives (n = 129 or 48%). Finally, where necessary the case notes and cause of death were reviewed.

RESULTS

Overall Results. Of the 1333 evaluable cases accrued in a 12-month period, 999 cases were assessed for both p53 status and cytopathological diagnosis. In 956 cases, a clear-cut cytopathological diagnosis had been made (Table 1). A diagnosis of malignancy was made for 205 cases; of these 86 (42%) showed overexpression of p53. In 22 specimens, nonneoplastic cells showed staining. There were 119 cases classified as false negatives (i.e., p53-negative nonneoplastic cells), although on review of the slides the tumor cells were considered to be markedly degenerate in 16 cases. Immunohistological examination of tumor tissue sections in 60 cases (50%) revealed that 20 were p53 positive, and 40 showed no detectable p53 staining. However, in 11 cases, <25% of the neoplastic cells were p53 positive. This emphasizes that some false negatives occur due to sampling of a tumor showing heterogeneous p53 expression; this would be particularly relevant where few tumor cells were present in the cytological sample.

False negatives also occur due to poor cellular preservation with loss of antigen, which can be a feature of some techniques by which cells are collected for cytological examination (e.g., sputa and bronchial washings; see below).

Of the 22 false positives (no tumor, p53 positive) 19 were due to positivity of reactive mesothelial cells; this was generally weak heterogeneous staining and in some cases was cytoplasmic as well as nuclear. The nature of staining was confirmed by immunostaining replicates with the polyclonal antiserum CM-1. Histological material was stained in 6 cases (2 pleural biopsies and 4 fluid clots), and none showed any detectable p53 overexpression. In 2 cases false positivity was due to weak, focal staining of the epithelial cells from ovarian cysts. The final case was an FNA sample from a fibroadenoma in which epithelial cells showed staining. The immunohistological examination of the subsequently excised lesion revealed positivity in <5% of the cells.

Of the 40 true positives for which histological material was stained, 26 (65%) were positive, and <25% of the tumor cells showed staining in 11 of these. For the cases which were negative on sections but positive on cytological preparations, a likely explanation is loss of antigenicity due to formalin fixation or processing to paraffin.

Analysis by Specimen Type. Of 227 urine specimens (Table 1) immunostained, 8 (3.5%) were diagnosed as neoplastic. Three (38%) of these exhibited p53 overexpression. From the 211 samples of serous fluids, 54 contained malignant cells, 36 (67%) of which were p53 positive. Staining of reactive mesothelial cells was observed in 19 cases. The study included 70 breast specimens of which 39 were cytologically malignant (56%); 15 (38%) of these showed p53 immunoreactivity. One fibroadenoma also showed p53 overexpression, and this was confirmed by histology in which <5% of cells stained. Of the 31 lymph node samples, 23 (74%) were neoplastic, of which 13 (56%) showed p53 staining. Only one of 5 (20%) lymphomas were positive, whereas 12 of 18 (63%) epithelial metastases showed p53 immunoreactivity. Four of 15 thyroid specimens were considered malignant: one follicular, two papillary, and one medullary carcinomas. The follicular carcinoma and one papillary carcinoma showed p53 overexpression, although staining was cytoplasmic as well as nuclear. Histology was available for all. However, only the papillary carcinoma was positive in 2 of the 5 blocks examined, and then only in <5% of neoplastic cells.

Of 30 ovarian cyst fluids, one was diagnosed as malignant and was p53 positive. There were 2 false positives, both being benign epithelial cells in which only occasional nuclei showed p53 immunoreactivity. There were 293 lung specimens studied. These were sputa (n = 168), bronchial washings (n = 104), and needle washings of lung biopsies (n = 17). In total, 47 (16%) were diagnosed as malignant, and overexpression of p53 was detectable in 10 cases (21%). Histological material was stained on 6 of the true positives, 5 of which showed overexpression (83%). Ten of 16 (56%) histological specimens corresponding to false negatives were positive. The results of positive p53 immunocytochemistry of sputa (8%, n = 12) and bronchial washings (17%, n = 24) compared with FNA samples (45%, n = 11)
show a significant difference ($P < 0.01$, $\chi^2$ test). This is presumably due to the nature of the cells collected in sputum samples and bronchial washings which yield degenerate "sloughed off" cells in which the p53 antigen is lost, unlike FNA samples which are fixed rapidly.

**Breakdown by Tumor Type.** Table 2 demonstrates that there are marked differences in the proportion of cases with detectable p53 expression with abnormalities being most prevalent in adenocarcinomas (51%), as has been previously reported (11–14).

**Cytologically Suspicious Cases.** Table 3 shows a contingency table analysis for this subgroup of specimens. In 43 cases the cytological diagnosis was equivocal with suspicion of neoplasia. These specimens comprised breast FNAs ($n = 7$), urine ($n = 11$), serous fluids ($n = 7$), lymph nodes ($n = 2$), sputa ($n = 7$), bronchial washings ($n = 3$), lung biopsies ($n = 2$), and miscellaneous ($n = 4$). Of these, 36 were p53 negative. However, in 7 cases suspicious cells showed nuclear p53 immunoreactivity. Analysis of subsequent histology and/or case notes indicated that all 7 were neoplastic (Table 4).

For the p53 negative cases, follow-up was available for 28; of these, 17 were neoplasms for which immunohistochemistry was positive in 3 of 10 cases.

**DISCUSSION**

The past decade has brought considerable advance in our understanding of the molecular basis of neoplasia (34) with the recognition that damage accumulates in key regulatory genes, known as oncogenes and tumor suppressor genes (35–37). While of considerable biological interest, there has to date been relatively little impact of this field on clinical practice. This has been principally for two reasons. First, the changes observed in any particular tumor type are often relatively specific, and such changes usually occur at relatively low frequency. Second, the methods required for the reliable detection of molecular alterations are often not simple and economical to apply to clinical samples. Consequently, to be of use clinically, tests must be devised that are easily and cheaply applied and that can be used in a wide range of tumor types. We have demonstrated the possible value of p53 immunostaining in diagnostic cytopathology and have shown in a large prospective series that there is an excellent correlation between expression of p53 immunoreactivity and malignancy and that the phenotype of p53 overexpression is seen in 42% of tumors. Previous reports (14, 28) have been retrospective in nature and based on selected series, while the current study is prospective and case selection unbiased. We have demonstrated that p53 immunostaining is a useful adjunct to conventional morphological diagnosis where the morphological appearances are suspicious but not diagnostic of malignancy. Of equal importance, we have identified types of samples and clinical situations for which p53 immunostaining is not of discriminant value.

When one considers the value of a new test, the presence of false-positive results (reflected in poor specificity) is of particular concern. In the case of p53 analysis there are now a number of important observations that indicate a biological basis for upregulation of p53 expression to levels detectable by conventional immunohistological methods. For example, the induction of p53 by genotoxic stress is now well defined (2–5). Other reports indicate that p53 staining can be detected in cells in the absence of mutation, although the mechanism is as yet unclear (38). We have observed a fibro-adenoma in which epithelial cells showed p53 immunoreactivity and other such cases have been reported (39, 40). The biological significance of this is uncertain. In experimental (41) and clinical material (42) there is very good concordance between overexpression of p53 in the majority of cells and molecular change in the p53 gene. If only a small proportion of cells stain, then this association is much weaker. This suggests that extreme caution should be used when interpreting p53 immunoreactivity when only small numbers of cells are stained (27). It should be noted that there are an increasing number of reports of benign neoplasms expressing detectable p53, typically only in occasional cells (43–46). In premalignant conditions this may represent mutation of p53 as an early step in the development of malignancy (43, 46).

The major source of false positives in our series has been in reactive mesothelial cells in which 19 of 22 samples showed nuclear staining. The nature of the staining was confirmed by use of a second anti-p53 antisera CM-1 (47) and has recently been reported by other workers (48). It is surprising then that no p53 immunoreactivity was demonstrated in histological material, although this is in accord with several other reports (49–51). It may be that detection thresholds (27) are important, with the relatively milder fixation methods used in cytology allowing the detection of p53, which after formalin fixation and embedding is undetectable. Specifically, we would emphasize that p53 overexpression is only present in a small proportion of mesothelial cells in any sample, as has been seen in other contexts (27). An alternative view may be that the growth arrest associated with cells in suspension (within a serous fluid) might induce increased p53 expression (52), whereas mesothelial cells embedded in tissue have low levels of p53. Whether reactive mesothelial staining represents ele-

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### Table 2 Incidence of p53 overexpression in different tumor types

<table>
<thead>
<tr>
<th>Tumor by histological type</th>
<th>p53 positive</th>
<th>p53 negative</th>
<th>Total</th>
<th>% positive</th>
</tr>
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<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>55</td>
<td>53</td>
<td>108</td>
<td>51</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>5</td>
<td>21</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Transitional cell carcinoma</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>4</td>
<td>12</td>
<td>16</td>
<td>25</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>7</td>
<td>10</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>Sarcomas</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* All non-Hodgkin's lymphomas.
* One Hodgkin’s and 4 non-Hodgkin’s lymphomas.

### Table 3 Contingency table for cytologically equivocal specimens

<table>
<thead>
<tr>
<th>Cytological diagnosis</th>
<th>p53 positive</th>
<th>p53 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytologically positive</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Cytologically negative</td>
<td>0</td>
<td>11</td>
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</table>

#### Table 4 Diagnoses and follow-up of cytologically equivocal specimens showing positive p53 staining

<table>
<thead>
<tr>
<th>Specimen nature</th>
<th>Cytological diagnosis</th>
<th>Histology result or follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland imprint</td>
<td>Phaeochromocytoma malignant?</td>
<td>Phaeochromocytoma malignant?</td>
</tr>
<tr>
<td>FNA of breast</td>
<td>Probably malignant</td>
<td>Ductal carcinoma</td>
</tr>
<tr>
<td>Urine</td>
<td>Suggestive of neoplasia</td>
<td>Transitional cell carcinoma</td>
</tr>
<tr>
<td>Ascites</td>
<td>Likely to be malignant</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>Suspicious</td>
<td>Small cell carcinoma</td>
</tr>
<tr>
<td>Lung biopsy</td>
<td>Probably adenocarcinoma</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>FNA of breast</td>
<td>Atypical phylloides or variant of a papillary lesion</td>
<td>Ductal origin carcinoma with sarcomatous metaplasia</td>
</tr>
</tbody>
</table>
DETECTION OF p53 PROTEIN IN CYTOLOGY

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REFERENCES

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