

File ID 61866  
Filename Chapter 5 Diagnostic p53 Immunostaining of Endobiliary Brush Cytology:  
Preoperative Cytology Compared with the Surgical Specimen

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SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type Dissertation  
Title Clinical significance of molecular markers in pancreatic cancer  
Author M. Tascilar  
Faculty Faculty of Medicine  
Year 2002  
Pages 106

FULL BIBLIOGRAPHIC DETAILS:

<http://dare.uva.nl/record/108583>

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## Chapter 5

### **Diagnostic p53 Immunostaining of Endobiliary Brush Cytology: Preoperative Cytology Compared with the Surgical Specimen**

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*Cancer Cytopathology* 1999; 87: 306-311

# Diagnostic p53 Immunostaining of Endobiliary Brush Cytology

## Preoperative Cytology Compared with the Surgical Specimen

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Supported by the Netherlands Foundation for Scientific Research (NWO), grant number 950-10-625, and by the Prevention Fund, grant number 28-2469.

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Received January 20, 1999; revision received April 13, 1999; accepted April 16, 1999.

**BACKGROUND:** Endobiliary brush cytology is important in the distinction of malignant and benign causes of extrahepatic bile duct obstruction. The additional diagnostic value of p53 immunostaining on these cytology specimens was assessed.

**METHODS:** All patients with extrahepatic bile duct obstruction who underwent endoscopic retrograde cholangiopancreatography (ERCP) with endobiliary brush cytology and subsequent surgery at the Academic Medical Center in Amsterdam during a 3-year period were studied. p53 Immunocytology was compared with the corresponding conventional light microscopic cytology and p53 immunostaining of the subsequent surgical specimen.

**RESULTS:** Fifty-three patients with the following diagnoses were included: pancreatic carcinoma (23), bile duct carcinoma (15), ampullary carcinoma (5), lymph node metastases (2), carcinoma of unknown origin (4), chronic pancreatitis (3), and primary sclerosing cholangitis (1). Fifty-one percent of the carcinomas showed positive p53 immunostaining; all four surgical specimens without carcinoma were negative. The sensitivities of conventional light microscopic cytology, p53 immunocytology, and both tests combined were 29%, 24%, and 43%, respectively. These sensitivities were higher in cases of bile duct carcinoma (46%, 40%, and 66%) compared with cases of pancreatic carcinoma (13%, 9%, and 22%). Specificities of both tests were 100%.

**CONCLUSIONS:** p53 Immunostaining on endobiliary brush cytology may be helpful in the diagnosis of malignant extrahepatic bile duct stenosis, especially in patients with bile duct carcinoma. *Cancer (Cancer Cytopathol)* 1999;87:306-11.

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**KEYWORDS:** ERCP, brush cytology, p53 immunocytochemistry, bile duct stenosis, pancreatic cancer, bile duct carcinoma.

Extrahepatic bile duct stenosis is caused by a variety of benign and malignant diseases. Symptomatology and diagnostic imaging techniques cannot readily differentiate between the two. Light microscopic tissue examination is needed to reach an unequivocal diagnosis of the cause of such an obstruction. For this purpose, brush cytology can be obtained during endoscopic retrograde cholangiopancreatography (ERCP). Unfortunately, although the specificity is almost 100%, the sensitivity of light microscopic evaluation of endobiliary brush cytology is only 30 to 40%.<sup>1,2</sup> The relatively low sensitivity is caused by the limited yield of material suitable for investigation, the difficulty in distinguishing epithelial cells with reactive changes from neoplastic cells, and the difficulty in distinguishing normal duct epithelium from highly differentiated carcinoma cells.

New potentially promising tumor markers detectable in brush cytology specimens come from molecular genetic cancer research. Various genetic alterations important in carcinogenesis have now been described, of which alterations in the *ras* oncogenes and the *p53* tumor suppressor gene are the most common. The diagnostic use of detection of *K-ras* mutations in biliary cytology has been reported with variable results, and it may be helpful in the diagnosis of malignant bile duct stenosis.<sup>3-15</sup> In contrast, the diagnostic use of detection of *p53* alterations in these cytology specimens has received only limited attention, mainly in pilot experiments.<sup>16-18</sup> Nevertheless, *p53* is a potentially useful target for diagnostic purposes.

The *p53* tumor suppressor gene is located on chromosome 17p and encodes for a nuclear transcription factor. The *p53* protein prevents the cell cycle from proceeding from G<sub>1</sub> to S-phase in cells with DNA damage, allowing DNA repair. The *p53* protein also plays a role in DNA repair itself and in apoptosis.<sup>19-21</sup> Thus, the cell loses three important controls of the cell life cycle when the *p53* protein is non-functional. Usually, loss of one allele and mutation of the other inactivate the *p53* tumor suppressor gene.

Alterations in the *p53* gene are attractive as a tumor marker in the diagnosis of malignant bile duct stenosis for the following reasons. Firstly, *p53* alterations are among the most frequent genetic alterations in human malignancies, including neoplasms causing bile duct stenosis.<sup>22</sup> The prevalence of *p53* mutations in carcinomas of the pancreas, bile duct, and ampulla of Vater is between 50 and 70%.<sup>23-25</sup> Secondly, *p53* immunocytochemistry (*p53*-IC) can be used as a surrogate test for time-consuming and cumbersome sequence analysis to detect mutations. Mutations in *p53* mostly lead to a conformational change of the protein product that has a prolonged half-life.<sup>26</sup> The mutant *p53* protein product accumulates in the nucleus, where it can be detected with simple, quick, and cheap immunochemical techniques available in routine laboratories. Immunostaining to detect *p53* is estimated to be 65 to 70% sensitive and 90% specific.<sup>27</sup> Finally, when immunocytochemical detection of *p53* mutations is used, the (cyto)pathological features remain intact for evaluation.

In this study we determined the diagnostic value of *p53* immunostaining of endobiliary brush cytology as an adjunct to conventional light microscopic cytology for the diagnosis of malignant extrahepatic bile duct stenosis. Brush cytology outcomes were compared with the results of the definitive surgical tissue specimens to evaluate possible reasons for discrepancies.

## MATERIALS AND METHODS

### Patients

All consecutive patients who underwent ERCP with brush cytology for the evaluation of an extrahepatic bile duct stenosis between 1993 and 1996 at the Academic Medical Center, Amsterdam, and who underwent subsequent surgery with resection of the stenotic lesion or biopsy of metastases were included if cytology smears and tissue of the surgical specimens were available for *p53* immunostaining.

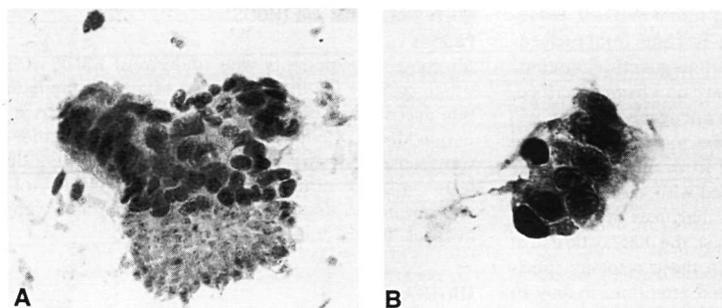
### MATERIALS

Brushings of the bile duct were performed with the GRBH-230-3-3.5 brush (Wilson-Cook Medical, Inc., Winston-Salem, NC). The brushes were washed in Roswell Park Memorial Institute (RPMI) 1640 (GibcoBRL, Rockville, MD) medium and immediately transported to the cytology laboratory. Four cytology smears or cytopins were made and stained with Giemsa and Papanicolaou for routine cytopathological diagnosis. Additional cytology smears were made, fixed with Pro-Fixx (Lerner-Laboratories, Pittsburgh, PA), wrapped in aluminium foil, and stored at -20 °C for subsequent *p53* immunostaining.

Five  $\mu$ m sections of formalin-fixed, paraffin-embedded tissue blocks were used for *p53* immunostaining.

### *p53* Immunostaining

Brush cytology smears were rinsed thoroughly in graded ethanol and distilled water. After endogenous peroxidase was blocked by methanol containing 0.3% peroxide, the slides were incubated in a 0.01 M sodium-citrate solution, pH 6.0, in a microwave oven set at 100 °C. After a 10-minute incubation period, the sections were allowed to cool for 30 minutes. After being rinsed twice in distilled water and phosphate-buffered saline (PBS), sections were treated by 20 minutes of incubation in 10% normal goat serum in PBS. The slides were then incubated 60 minutes in a 1:1000 solution of CM1, a rabbit polyclonal antibody against the *p53* protein (Novocastra, Newcastle upon Tyne, UK). Biotinylated swine anti-rabbit immunoglobulins were used as secondary antibody and were applied in a 1:400 solution with 10% pooled human AB-serum for 30 minutes. The next step contained streptavidin (1:200) with biotinylated horseradish peroxidase (1:200, Dakopatt, Denmark) in PBS with 10% pooled human AB-serum, which was applied for 30 minutes. Chromogen was 5% diaminobenzidine (DAB) and substrate was 0.03% peroxide in TRIS (tris-hydroxymethyl-amino methane)-HCl 0.05M, pH 7.8. A 10-minute incubation time resulted in a brown precipitate in the nuclei of cells from a colon carcinoma with a known



**FIGURE 1.** Positive p53 immunostaining of endobiliary brush cytology; note the specific staining of the malignant cells (A;  $\times 100$ ). (B) A positive result of conventional light microscopy (Papanicolaou staining,  $\times 160$ ).

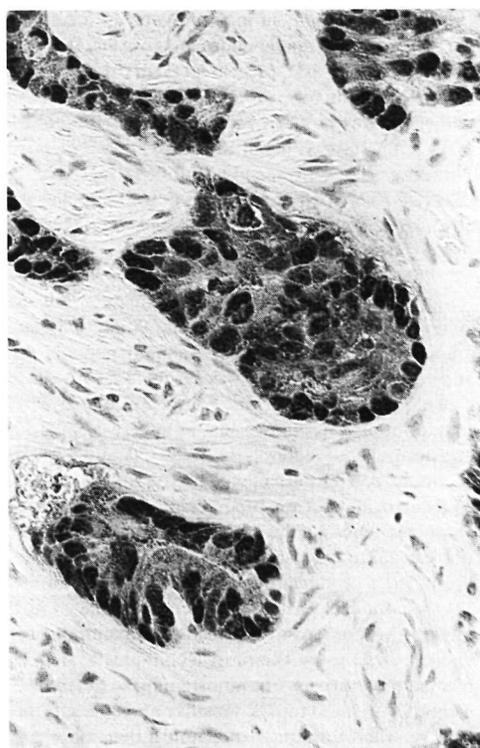
p53 mutation that was used as a positive control. Nuclear counterstaining was done with hematoxylin. As a negative control, part of each specimen followed the whole procedure leaving out the primary antiserum.

Tumor sections were mounted on organosilan-coated glass slides (Menzel-Gläser, Germany) and dried overnight at 37 °C. Sections were dewaxed in xylene and graded ethanol, after which they were placed in a coplin jar filled with 0.3% peroxide in methanol. Subsequently, the slides were processed as described above.

All cytological and histological samples were coded and evaluated independently by two observers. Brush cytology specimens were considered p53 immunocytochemical-positive if one or more cells, recognizable as epithelial cells, showed unequivocal nuclear staining. Tissue was considered p53 immunohistochemical-positive if at least 10% of the tumor cells showed specific nuclear staining<sup>25</sup> (Figs. 1A and 2).

#### Light Microscopy

The routine diagnostic cytology smears were coded and reviewed (Fig. 1B). For the purpose of this study they were reclassified as positive for malignancy, negative for malignancy, suspicious for malignancy, or not suitable/insufficient for diagnosis. For the purpose of this study we have deliberately avoided the term "atypical" in the classification, because it is a descriptive light microscopic term that can be applied both for reactive and neoplastic atypia, and it is therefore not unambiguous. Thus, we classified the cytology results either as positive, when the atypia was indicative of malignancy, or as negative, when the atypia was interpreted as consistent with reactive cells. In those cases in which there was uncertainty whether the atypia should be considered as neoplastic or reactive, we classified them as suspicious.



**FIGURE 2.** Positive p53 immunohistochemistry of the bile duct carcinoma corresponding to Figure 1.

#### RESULTS

Fifty-three patients were included in the study (Table 1). The mean age was 58 years, and 32 were males. The final histological diagnoses were as follows: 23 pancreatic carcinomas, 15 bile duct carcinomas, 5 amp-

**TABLE 1**  
**Diagnosis, p53 Immunostaining of Surgical Specimens, p53 Immunocytology, and Conventional Cytology in 53 Patients with Extrahepatic Bile Duct Stenosis**

Diagnosis	Number	p53-IC-positive surgical specimens (%)	Positive conventional cytology (%)	p53-IC-positive cytology (%)	Positive conventional cytology and/or p53-IC-positive cytology (%)
Pancreatic carcinoma	23	11 (48)	3 (13)	2 (9)	5 (22)
Bile duct carcinoma	15	8 (53)	7 (46)	6 (40)	10 (66)
Ampullary carcinoma	5	2 (40)	1 (20)	—	1 (20)
Lymph node metastasis	2	2 (100)	1 (50)	2 (100)	2 (100)
Carcinoma unspecified	4	2 (50)	2 (50)	2 (50)	3 (75)
Chronic pancreatitis	3	—	—	—	—
Primary sclerosing cholangitis	1	—	—	—	—

**TABLE 2**  
**p53 Immunocytochemistry Results of Surgical Specimens (Histology) and Cytology Specimens Compared with Conventional Cytology Results in the 49 Patients With Malignant Extrahepatic Bile Duct Stenosis**

p53-IC results	No. of patients	Conventional cytology results		
		Positive	Negative	Suspicious
Histology+/cytology-	9	4	4	1
Histology-/cytology+	3	1	1	1
Histology+/cytology-	16	2	8	6
Histology-/cytology-	21	7	12	2
Total	49	14	25	10

ullary carcinomas, 2 lymph node metastases (1 lung carcinoma and 1 rectal carcinoma), 3 chronic pancreatitis, and 1 primary sclerosing cholangitis. Four patients were diagnosed with "unspecified carcinoma." In these patients biopsies from the metastases only were obtained, and thus no specific diagnosis as to the tissue of origin could be established.

Fifty-one percent of the surgical specimens with carcinoma were p53-IC-positive: 48% of the pancreatic carcinomas, 53% of the bile duct carcinomas, 40% of the ampullary carcinomas, 50% of the unspecified carcinomas, and both the lymph node metastases. All four surgical specimens from patients with benign stenoses were p53-IC negative.

Of the 49 patients with malignant bile duct stenosis, 14 were accurately diagnosed with conventional cytology (positive cytology, suspicious not included), and 12 were diagnosed with p53 immunocytochemistry (positive p53 immunocytochemistry). Of the 12 diagnosed by p53 immunocytochemistry, 7 were not accurately diagnosed with conventional cytology (Table 2). Sensitivities of conventional cytology, p53 immunocytochemistry, and both tests combined were 29%, 24%, and 43%, respectively. The sensitivities were higher for bile duct carcinoma (46%, 40%, and

66%) compared with pancreatic carcinoma (13%, 9%, and 22%). None of the cytology specimens from the four patients with benign bile duct stenoses had positive results for conventional cytology or p53 immunocytochemistry (specificity 100%).

The p53 immunocytochemistry results of the cytology specimens and the surgical specimens were concordant in 34 (64%) patients, including the 4 patients with benign stenoses. Sixteen patients with p53-IC-positive carcinomas had negative p53 immunocytochemistry results, and 3 patients with p53-IC-negative carcinomas had positive p53 immunocytochemistry results (Table 2).

**DISCUSSION**

The detection of cancer-specific molecular alterations as a diagnostic adjunct to diagnostic cytology specimens is attractive because of the often low sensitivity of conventional light microscopic examinations. Prerequisites for a successful diagnostic marker are a high prevalence of the genetic alteration in the specific neoplasm and a detection method that is relatively simple. Both prerequisites hold true for p53 alterations in malignancies causing bile duct stenosis: the p53 gene is mutated in 50 to 70% of these malignancies, and the detection of the mutant p53 protein product with standard immunocytochemical procedures is in general representative for mutations in p53<sup>23-27</sup>.

The frequency of positive p53 immunohistochemistry for the different carcinomas corresponds with that of previous reports<sup>23-25</sup>, indicating that our study group is representative. The sensitivity of conventional cytology was 29%, which is also in line with previous large studies on endobiliary brush cytology<sup>1,2</sup>. The sensitivity of p53 immunocytochemistry alone was 24%, which is relatively low. However, the combined sensitivity of both tests was 43%, an increase of 14% above that of the conventional cytology alone. Thus, although of limited diagnostic value, p53

immunocytochemistry certainly adds to conventional methodology. The specificity of both tests was 100%, but the number of patients without malignancy was small. Mutations of *p53* are not described in non-malignant lesions, but false-positive *p53* immunostaining may occur<sup>28,29</sup>.

Absence of *p53* overexpression in the primary tumor was the major cause for the limited diagnostic value of *p53* immunocytochemistry (21 cases). Another cause was sampling error, i.e., the absence of tumor cells in the cytology specimens as reflected by the negative or inconclusive conventional cytology results in 14 of the 16 patients with *p53*-IC-positive tumors who had negative *p53* immunocytochemistry. The two patients with negative *p53* immunocytochemistry but *p53*-IC-positive tumors and positive conventional cytology results can be considered to have "true" false-negative staining of the cytology samples. This may be the result of enzymatic influences of bile products, or it may be because of technical error<sup>29</sup>. Intratumor heterogeneity may be an alternative explanation for these discrepancies.

Intratumor heterogeneity has been demonstrated in many tumors, not only as far as morphological characteristics are concerned but also regarding cytogenetic aberrations ranging from large chromosomal abnormalities to point mutations in genes such as *p53*<sup>14,30-36</sup>. The three cases with the discrepant findings of a *p53*-IC-negative tumor and *p53*-IC-positive cytology may also be explained by the presence of intratumor heterogeneity for *p53* overexpression. Unfortunately, there was no additional tissue of the primary tumor available for immunostaining to explore whether this was indeed the case. However, in a previous study we were able to demonstrate that intratumor heterogeneity for *p53* immunohistochemical staining may account for non-concordant *p53* immunocytochemistry<sup>14</sup>.

Higher sensitivities than were found in this study have been reported for both conventional cytology and *p53* immunocytochemistry for the diagnosis of pancreatic cancer<sup>17,18</sup>. This can be explained by selection resulting from different inclusion criteria used in these studies. The authors examined a series of patients with pancreatic carcinoma or chronic pancreatitis in whom selective brushing of the pancreatic duct stenosis was performed. Thus, patients were selected on the basis of an established diagnosis and the ability to pass the brushing device through the pancreatic duct, which can be difficult in certain cases. Our study is essentially different. It included a consecutive series of patients each with a prominent extrahepatic bile duct stenosis from which endobiliary brush cytology was obtained preoperatively to differentiate between a

malignant or benign cause of the bile duct stenosis. This is, in fact, the clinical setting most often encountered and for which additional molecular markers would be of great importance.

In cases of pancreatic carcinoma, the bile duct stenosis may be caused by external compression from the tumor rather than by direct transmural growth. As a result, in pancreatic cancers the tumor is not brushed directly, in contrast to what occurs with bile duct carcinomas, and the yield of tumor cells is low. Both conventional cytology and *p53* immunocytochemistry depend on the yield of malignant cells and the quality of these cells in the brush cytology specimens. This is illustrated by the higher sensitivity of conventional cytology and *p53* immunocytochemistry for the diagnosis of carcinoma arising from the bile duct itself compared with the diagnosis of pancreatic carcinoma<sup>37</sup>. Forty percent of the bile duct carcinomas were diagnosed with *p53* immunocytochemistry versus 9% of the pancreatic carcinomas. Also, the additional diagnostic value was higher in bile duct carcinoma than in pancreatic cancer, 20% versus 9%.

More sensitive polymerase chain reaction-based techniques to detect cancer-specific molecular alterations probably have more potential to increase the diagnostic yield of cytology specimens, but most of these techniques are still too complicated to use on a large scale within a routine clinical setting. In individual cases, additional *p53* immunocytochemical staining performed on endobiliary brush cytology specimens may certainly be helpful in determining further diagnostic or therapeutic strategies in patients with extrahepatic bile duct stenoses, particularly in patients with bile duct carcinoma.

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