K-ras and p53 in cancer of the pancreas and extrahepatic biliary tract

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Diagnosis p53 Immunostaining of Endobiliary Brush Cytology: Preoperative Cytology Compared to Surgical Specimen


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ABSTRACT
Endobiliary brush cytology is important in the differentiation between extrahepatic bile duct stenosis caused by malignant and benign disorders. The additional diagnostic value of p53 immunostaining on these cytology specimens was assessed.

All patients with an extrahepatic bile ducts stenosis who underwent endoscopic retrograde cholangiopancreaticography with endobiliary brush cytology and subsequent surgery at the Academic Medical Center in Amsterdam in a 3-year period were included if cytology specimens were suitable for p53 immunostaining. P53 immunocytoLOGY was compared with the corresponding conventional cytology and p53 immunostaining of the surgical specimens.

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

P53 immunostaining of endobiliary brush cytology can be helpful in the diagnosis of malignant extrahepatic bile duct stenosis, especially in patients with bile duct carcinoma.
Introduction

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

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 ones. 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 [3-14]. In contrast, the diagnostic use of detection of p53 alterations in these cytology specimens has only received limited attention, mainly in pilot experiments [15-17]. P53 is nevertheless 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 to proceed from G1 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 [18-20]. Thus, the cell loses 3 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 one of the most frequent genetic alterations in human malignancies including neoplasms causing bile duct stenosis [21]. The prevalence of p53 mutations in carcinoma of the pancreas, bile duct, and ampulla of Vater is between 50 and 70% [22-24]. Secondly, p53 immunostaining can be used as a surrogate test for time-consuming and cumbersome sequence analysis to detect mutations. P53 mutations mostly lead to a conformational change of the protein product that has a prolonged half-life [25]. The mutant protein product accumulates in the nucleus where it can be detected with simple, quick, and cheap immunohistochemical techniques available in routine laboratories (p53 overexpression). Immunostaining to detect p53 mutations is estimated to be 65-70% sensitive and 90% specific [26]. Finally, when immunohistochemical 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 (Wilson-Cook Medical Inc.). The brushes were immediately transported to the pathology department. Four cytology smears or cytospins 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, USA), wrapped in aluminum foil, and stored at -20 °C for subsequent p53 immunostaining.

Five μm sections of formalin-fixed paraffin-embedded tissue blocks were used for p53 immunostaining.

P53 Immunostaining
Brush cytology smears were rinsed thoroughly in distilled water and incubated in a 0.01M natrium-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 down for 30 minutes. After rinsing twice in distilled water and phosphate buffered saline (PBS), sections were treated by a 20 minutes incubation in a 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 was used as secondary antibody and was applied in a 1:400 solution with 10% AB-serum for 30 minutes. The next step contained strept. ABC Hrp 2% (Dakopatt, Denmark) in PBS with 10% AB-serum and was applied for 30 minutes. Chromogen was 5% diaminobenzidine (DAB) and substrate was 0.03% peroxide in Tris-HCl 0.05M, pH 7.8. A 10 minutes incubation time resulted in a brown precipitate in the nuclei of cells from a colon carcinoma with a known p53 mutation that was used as a positive control. Nuclear counterstaining was done with haematoxilin. As a negative control, part

of each specimen followed the whole procedure leaving out the primary antiserum.

Tumor sections were mounted on organosilicon coated glass slides and dried overnight at 37 °C. Sections were de-waxed in xylene and graded ethanol, after which they were placed in a coplin jar filled with 0.3% peroxidase in methanol. Subsequently, the slides were processed as described above.

All cytological and histological samples were coded and evaluated by two independent observers. Brush cytology specimens were considered p53 immunohistochemical (p53 IC)-positive if one or more cells, recognizable as epithelial cells, showed unequivocal nuclear staining. Tissue was considered p53 IC-positive if at least 10% of the tumor cells showed specific nuclear staining [24] (Figure 6).

Light Microscopy
The routine diagnostic cytology smears were coded and reviewed for this study. They were classified as positive for malignancy, negative for malignancy, suspicious for malignancy, or not suitable/insufficient for diagnosis.

Results
Fifty-three patients were included in the study (Table 1). The mean age was 58 years, and 32 were males. The following diagnoses were made: 23 pancreatic carcinomas, 15 bile duct carcinomas, 5 ampullary carcinomas, 2 lymph node metastases (one lung carcinoma and one rectal carcinoma), 3 chronic pancreatitis and 1 primary sclerosing cholangitis. Four patients were diagnosed with 'unspecified carcinoma'. In these patients only biopsies from the metastases 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,
TABLE 1
Diagnosis, p53 immunostaining of surgical specimens, p53 immunocytology, and conventional cytology in 53 patients with extrahepatic bile duct stenosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>p53 IC positive surgical specimens(%)</th>
<th>Positive conventional cytology(%)</th>
<th>p53 IC positive cytology (%)</th>
<th>Positive conventional cytology and/or p53 IC positive cytology(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic carcinoma</td>
<td>23</td>
<td>11 (48)</td>
<td>3 (13)</td>
<td>2 (9)</td>
</tr>
<tr>
<td>Bile duct carcinoma</td>
<td>15</td>
<td>8 (53)</td>
<td>7 (46)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Ampullary carcinoma</td>
<td>5</td>
<td>2 (40)</td>
<td>1 (20)</td>
<td>-</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>2</td>
<td>2 (100)</td>
<td>1 (50)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Carcinoma unspecified</td>
<td>4</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
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(13%, 9%, and 22%). None of the cytology specimens from the 4 patients with a benign bile duct stenosis had positive results for conventional cytology or p53 immunocytology (specificity 100%).

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

TABLE 2
P53 immunochemistry results of surgical specimens and cytology specimens, and conventional cytology compared in the 49 patients with malignant extrahepatic bile duct stenosis

<table>
<thead>
<tr>
<th>p53 IC results</th>
<th>Conventional cytology results</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor+/cytology+</td>
<td>Pos.</td>
<td>Neg.</td>
</tr>
<tr>
<td>Tumor-/cytology+</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Tumor+/cytology-</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Tumor-/cytology-</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>14</td>
</tr>
</tbody>
</table>

40% of the ampullary carcinomas, 50% of the unspecified carcinomas, and both the lymph node metastases. All 4 surgical specimens from patients with a benign stenosis were p53 IC negative.

Of the 49 patients with malignant bile duct stenosis 14 were accurately diagnosed with conventional cytology, and 12 were diagnosed with p53 immunocytology. Through p53 immunocytology, 7 patients were diagnosed in addition to the conventional cytology. Sensitivities of conventional cytology, p53 immunocytology, and both tests combined were 29%, 24%, and 43%, respectively. The sensitivities were higher for bile duct carcinoma (46%, 40%, and 66%) compared to pancreatic carcinoma (13%, 9%, and 22%).

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 morphological examinations. In order to be successful as a diagnostic marker, the genetic alteration should be frequently encountered in the carcinoma under diagnosis and the detection method should be relatively simple. Both prerequisites hold true for p53 alterations in malignancies causing bile duct stenosis. The p53 gene is mutated in 50-70% of these malignancies and the detection of the mutant p53 protein product with standard immunochemical procedures is in general representative for mutations in p53 [22-26].

The frequency of positive p53 im-
munostaining for the different carcinomas corresponded with previous reports [22-24], indicating that the study group can be considered representative. The sensitivity of conventional cytology was 29%, which is also in line with previous large studies on endobiliary brush cytology [1,2]. The sensitivity of p53 immunocytology 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 immunocytology certainly adds to conventional methodology. The specificity of both tests was 100%, but the number of patients without malignancy was small. P53 mutations are not described in non-malignant lesions, but false positive p53 immunostaining occurs [27-28].

Absence of p53 overexpression was the major cause for the limited diagnostic value of p53 immunocytology (21 cases). Another cause was likely the absence of tumor cells in the cytology specimens as reflected by the negative or inconclusive conventional cytology results in 14 of the 16 cases with p53 IC-positive tumors and negative p53 immunocytology. The 2 cases with negative p53 immunocytology but p53 IC-positive tumors and positive conventional cytology concern 'true' false negative staining of the cytology samples. This may be the result of enzymatic influences of bile products or due to technical error [28]. 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 [14,29-35]. The 3 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 available to demonstrate that this was the case. However, in a previous study we were indeed able to demonstrate that intratumor heterogeneity for p53 immunostaining may account for 'false positive' cytology [14].

Higher sensitivities have been reported for both conventional cytology and p53 immunocytology for the diagnosis of pancreatic cancer [16-17]. This can be explained by different inclusion criteria used in these studies. The authors examined a series of patients with pancreatic carcinoma or chronic pancreatitis in which selective brushing of the pancreatic duct stenosis was performed. Thus, patients were selected on an established diagnosis, and the possibility to pass the brushing device through the pancreatic duct which can be difficult in certain cases. Our study is essentially different. A consecutive series of patients were included with a prominent extrahepatic bile duct stenosis from which endobiliary brush cytology preoperatively was obtained to differentiate between a malignant or benign cause of the bile duct stenosis. This is in fact the clinical setting mostly encountered and for which additional molecular markers would be of great importance.

In case of pancreatic carcinoma, the bile duct stenosis may be caused by external compression from the tumor rather than direct transmural growth. As a result the tumor is not brushed directly in contrast to bile duct carcinomas and the yield of tumor cells will be low. Both, conventional cytology and p53 immunocytology will be dependent 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 immunocytology for the diagnosis of carcinoma arising from the bile duct itself as compared to pancreatic carcinoma [36]. Forty percent of the bile duct carcinomas were diagnosed with p53 immunocytology versus 9% of the pancreatic carcinomas. Also, the additional diagnostic value was higher in bile duct carcinoma, 20% versus 9%.

More sensitive PCR based techniques to detect cancer specific molecular alterations have probably 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 immunostaining performed on endobiliary brush cytology may certainly be helpful in determining further diagnostic or therapeutic strategies in patients with extrahepatic bile duct stenosis, particularly in patients with bile duct carcinoma.

References

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Can K-ras Codon 12 Mutations Be Used to Distinguish Benign Bile Duct Proliferations from Metastases in the Liver?
A Molecular Analysis of 101 Liver Lesions from 93 Patients


American Journal of Pathology 1997; 151:943-949

ABSTRACT
It can be difficult to distinguish benign bile duct proliferations (BDPs) from well-differentiated metastatic peripancreatic adenocarcinomas on histological ground alone. Most peripancreatic carcinomas harbor activating point mutations in codon 12 of the K-ras oncogene, suggesting that K-ras mutational status may provide a molecular basis for distinguishing BDPs from liver metastases. The ability of tests for mutations in codon 12 of K-ras to make this distinction was examined in a two-part study.

In the first part we determined the K-ras mutational status of 56 liver lesions and 48 primary peripancreatic adenocarcinomas obtained from 48 patients. In the second part of this study an additional 45 liver lesions were studied.

In the first 48 patients, activating point mutations in codon 12 of the K-ras were detected in 28 (61%) of the 46 primary carcinomas, in 8 (100%) of 8 liver metastases, in 2 (6.5%) of 31 BDPs, and in none (0%) of 14 liver granulomas. Three BDPs from 3 of the 48 patients did not amplify. Two of these were therefore excluded from further analysis, the third patient had a second liver lesion and remained in the series. To further estimate the prevalence of K-ras mutations in BDPs we analyzed an additional series of 45 mostly incidental BDPs for K-ras mutations. Three (6.7%) of these 45 harbored K-ras mutations.

These results suggest that K-ras mutations may be useful in distinguishing BDPs from metastases in the liver; however, there is some overlap in the mutational spectra of BDPs and pancreatic carcinomas.
CHAPTER 5

Can K-ras Codon 12 Mutation Be Used to Distinguish Benign Bile Duct Proliferations from Metastases in the Liver?

A Molecular Analysis of 101 Liver Lesions from 69 Patients

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