Genes and surgery in pancreatic cancer
van Heek, N.T.

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CHAPTER 2
K-ras, p53, and DPC4 (MAD4) alterations in fine-needle aspirates of the pancreas

A molecular panel correlates with and supplements cytologic diagnosis
Background
Although fine-needle aspiration is a useful technique in the diagnosis of pancreatic cancer, a significant number of cytological specimens cannot definitively diagnose or completely rule out adenocarcinoma. Because most pancreatic adenocarcinomas show alterations in the K-ras, p53, and DPC4 genes, we hypothesized that a genetic panel of these three genes may supplement traditional cytological analysis.

Methods
Between January 1997 and February 2000, 101 fine-needle aspirates of the pancreas were prospectively collected. After a cytologic diagnosis was made, possible molecular alterations were determined on the 94 aspirates with adequate tissue using a panel consisting of the K-ras, p53, and DPC4 (MAD4) genes.

Results
Of the included 94 fine-needle aspirates of the pancreas, 48 (51%) were diagnostic of adenocarcinoma, 19 (20%) were atypical (suggestive of, but not diagnostic of, adenocarcinoma), 25 (27%) were negative for adenocarcinoma, and 2 (2%) were diagnostic of a neoplasm other than adenocarcinoma. Clinical follow-up revealed that three patients (12%) with negative cytologic diagnoses and 12 patients (63%) with atypical cytologic diagnoses had adenocarcinoma. Of 63 with a final diagnosis of adenocarcinoma, 42 (67%) had an alteration in at least one of the genes analyzed. In contrast, only 2 (6%) of 31 patients without adenocarcinoma had an alteration in one gene of the panel. Overall, the molecular analyses supported the diagnosis of adenocarcinoma in 6 (32%) of 19 aspirates originally diagnosed as atypical by cytology alone.

Conclusions
A molecular panel that includes the K-ras, p53, and DPC4 (MAD4) genes correlates with and can supplement traditional cytologic diagnosis of pancreatic fine-needle aspirates.
INTRODUCTION

Pancreatic cancer is a genetic disease with a characteristic molecular genetic profile. The genetic alterations in pancreatic cancer include the activation of an oncogene, K-ras, and inactivation of tumor-suppressor genes, including p53 and DPC4 (MAD4). K-ras is activated in approximately 95% of pancreatic ductal adenocarcinomas, and the p53 and DPC4 (MAD4) genes are inactivated in 70% and 55% of cases, respectively. While alterations in K-ras and p53 occur often in many other types of neoplasms, inactivation of the DPC4 (MAD4) tumor-suppressor gene is relatively specific for ductal adenocarcinoma of the pancreas.

Activation of the K-ras gene is easily assessed genetically, because the vast majority of mutations occur in codon 12. The p53 and DPC4 (MAD4) genes are harder to study genetically. This is because they are inactivated in cancer, either by intragenic mutation in one allele coupled with loss of the second allele (loss of heterozygosity) or by deletion of both alleles (homozygous deletion). Because pancreatic adenocarcinomas evoke intense nonneoplastic desmoplastic responses, most of the DNA contained in a tumor is actually nonneoplastic DNA. This intimate admixture of normal and neoplastic tissues can mask the loss of a p53 or DPC4 (MAD4) allele in a cancer. A simple way to overcome this problem is to study these genes immunohistochemically. Immunohistochemical stains for the p53 and DPC4 (MAD4) proteins have recently become available, and, thus, alterations in these genes have become much easier to recognize.

Thus, genetic and immunohistochemical techniques have provided much information about the molecular biology of pancreatic ductal adenocarcinoma. However, there have been only a few studies examining the diagnostic usefulness of detecting these molecular changes in fine-needle aspirates of the pancreas. In addition, while K-ras and p53 alterations have been identified in fine-needle aspirates of the pancreas, no group has yet studied inactivation of the DPC4 (MAD4) gene in these specimens. Therefore, we set out to assess K-ras, p53, and DPC4 (MAD4) alterations in fine-needle aspiration specimens of the pancreas. We did this with two goals in mind: (1) to determine whether this molecular panel correlates with cytologic diagnosis and (2) to explore whether the use of this molecular panel could support the diagnosis of cancer in cytologic samples interpreted as atypical (suggestive of, but not diagnostic of, adenocarcinoma). In cases of atypical cytology, additional diagnostics, such as biopsy or laparotomy, are necessary to exclude a carcinoma. The morbidity and mortality attending additional diagnostics could be avoided if the use of a molecular panel could help support a benign or malignant diagnosis in patients who have atypical aspirates.
MATERIALS AND METHODS

Specimen selection

Between mid-January 1997 and mid-February 2000, 101 fine-needle aspirates of the pancreas, each from a different patient, were obtained prospectively at Oregon Health Sciences University, Portland. A routine clinical diagnosis was made on each of these cases at the time of the procedure by one of a group of pathologists at this institution. These cases were then collected and rereviewed by one cytopathologist (AER). Based on diagnoses made on rereview of Papanicolaou- and rapid Romanowsky-stained smears and H&E-stained cell-block sections, each specimen was grouped into one of four categories: (1) diagnostic for adenocarcinoma, (2) suggestive of adenocarcinoma, (3) no evidence of malignancy, and (4) diagnostic of neoplasm other than adenocarcinoma (figure 1). This categorization was performed before molecular analysis began.

Figure 1 Three representative fine-needle aspirates of the pancreas. The first (A) was diagnosed as “diagnostic of adenocarcinoma,” and the second (B) was “negative for malignancy.” The third aspiration (C) contains atypical cells that are suggestive, but not diagnostic, of malignancy. (Papanicolaou; A, ×600; B and C, ×400) (see page 257)
Data procurement and statistical analysis
Clinical and pathologic data for each case were obtained from patients' medical records and the Social Security Death Index database (available at http://www.ancestry.com). Once these data were acquired for each case, each specimen was given a unique identification number that could not be traced to the original cytology specimen number. Thus, molecular analysis was performed in a blinded manner, without connection to patient identifiers. This protocol was reviewed and approved by the Oregon Health Sciences University institutional Review Board. Cross-tabulations were analyzed with the chi-square or Fisher exact test, where appropriate. Means were compared with the t test. Each of these tests was 2-tailed.

K-ras gene analysis
Six unstained 5-μm slides were cut from cell blocks corresponding to each specimen. In 7 cases there were not enough cells present for subsequent analysis on some or all of the slides. These cases were excluded from the study, so 94 fine-needle aspirates remained to be studied. One of the six unstained slides was stained with H&E for comparison with the original cell-block slide on which a diagnosis had been made. From one to three of the unstained slides, depending on the number of cells on each slide, cells were scraped off and used for independent K-ras gene analyses. Two of the remaining slides were used for p53 and DPC4 (MAD4) immunohistochemical analysis. The microdissected tissue for K-ras gene analysis was placed in 50-μL DNA isolation buffer (10-mmol/L concentration of tris(hydroxymethyl)aminomethane hydrochloride at pH = 8, 0.2% polysorbate 20, and 100 μg/mL of Proteinase K). The mixture was incubated at 56°C for 16 to 18 hours, and the Proteinase was inactivated at 95°C for 10 minutes. The mixture was stored at −20°C until use. The mixtures were screened for point mutations in codon 12 of the K-ras gene, according to a modification of a previously described protocol. First, DNA from each specimen was amplified by polymerase chain reaction (PCR) for 15 cycles with primers 1 (5' ACT GA A TA T AA A CT T GT C GT A GT T GG A CC T 3') and 2 (5' TC A TG A AA A TG G TC A GA G AA A C C 3'). The PCR product was then split into two equal portions. One of the two portions was digested with Mval (Boehringer-Mannheim, Mannheim, Germany). Both portions then were amplified with primers 1 and 3 (5' TCA TGA AAA TGG TCA GAG AAA CC 3'). Because Mval cleaves wild-type but not mutant K-ras, an unenriched sample and a sample enriched for mutant K-ras were produced. Finally, each set of PCR products was denatured at 95°C for 10 minutes, spotted onto 7 different nylon membranes (Gene-Screen Plus, NEN Research Products, Boston, MA), and hybridized to each of 7 phosphorus 32-labeled, sequence-specific oligodeoxynucleotide probes. A final stringency wash at 63°C and autoradiography were carried out. PCR products amplified from plasmid clones containing each of 7 possible sequences at codon 12 were used as positive controls on the hybridization filters. Negative control samples (water only) were included throughout the series.
**p53 and DPC4 (MAD4) immunohistochemical analysis**

Two unstained 5-μm slides were deparaffinized by routine techniques. The slides were treated with sodium citrate buffer (diluted to 1× from 10× HIER buffer, Ventana-Bio Tek Solutions, Tucson, AZ) and then steamed for 20 minutes at 80°C. After cooling for 5 minutes, one slide was labeled with monoclonal antibody to p53 (antibody DO-7, 1:500 dilution, DAKO, Carpinteria, CA), and the second slide was labeled with monoclonal antibody to DPC4 (MAD4) (clone B8, 1:100 dilution, Santa Cruz, Santa Cruz, CA). A Bio Tek-Mate 1000 automated stainer (Ventana-Bio Tek Solutions) was used to perform the labeling. The antibodies were detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin. Four of us (NTvH, GJAO, RHH, REW) evaluated the immunohistochemical labeling in each of the cases. The labeling in each case was scored as “positive” or “negative.” Because mutations in the p53 protein result in longer-lived proteins, positive labeling for p53 suggests a p53 alteration.26 Cases were scored as showing positive labeling for p53 when there was unequivocal and specific nuclear expression of p53 in one or more cells. Because mutations in or deletions of DPC4 (MAD4) usually result in the truncation or absence of the protein, loss of labeling for DPC4 (MAD4) indicates a DPC4 (MAD4) alteration.24 Cases scored as showing loss of labeling for DPC4 (MAD4) had complete loss of labeling in both the cytoplasm and nuclei of cells, compared with other positive cells on the slide, which served as internal controls. Thus, because one looks for overexpression of protein in the p53 assay, all of the cells on a given slide were evaluated for p53. In contrast, in the Dpc4 assay, only cells with epithelial atypia, as compared with normal-appearing positive controls, were evaluated for Dpc4 loss. The interpretation of immunohistochemical labeling of the cancers was highly robust, with agreement among the observers in all cases. Sections of normal pancreas, which show negative p53 and positive DPC4 (MAD4) staining, served as positive controls in each immunohistochemical run.

**Cytology and patient characteristics**

Based on the initial review of the cytologic material, 48 (51%) of the 94 fine-needle aspirates included in this study were diagnostic of adenocarcinoma, 19 (20%) were suggestive of adenocarcinoma, 25 (27%) were negative for malignancy, and 2 (2%) were diagnostic of a neoplasm other than adenocarcinoma (Table 1). One tumor in the latter category was a solid-pseudopapillary neoplasm; this patient was alive 11 months after her diagnosis was made. The other tumor was diagnosed on cytology as “consistent with either mucinous cystic neoplasm or intraductal papillary mucinous neoplasm”; the patient with this mucinous tumor died four months after his initial diagnosis. The 94 patients included in the study were followed up for median and mean periods of 15 and 16 months, respectively. The follow-up data were obtained by reviewing the patients' medical
Table 1 Correlation between cytologic and molecular diagnoses*

<table>
<thead>
<tr>
<th>Cytologic diagnosis</th>
<th>Molecular diagnosis</th>
<th>Mutant K-ras</th>
<th>p53 Labeling</th>
<th>Loss of Dpc4 Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic for adenocarcinoma (n = 48)</td>
<td></td>
<td>24 (50)</td>
<td>25 (52)</td>
<td>11 (23)</td>
</tr>
<tr>
<td>Suggestive of adenocarcinoma (n = 19)</td>
<td></td>
<td>3 (16)</td>
<td>5 (26)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Negative for malignancy (n = 25)</td>
<td></td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Diagnostic for neoplasm, not adenocarcinoma (n = 2)</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage)

records and verifying their survival status with the Social Security Death Index database. Clinical follow-up revealed that all of the 48 patients with malignant cytologic diagnoses had evidence of adenocarcinoma, while 3 (12%) of the 25 patients with negative cytologic diagnoses had adenocarcinoma. In the former group, 16 patients had histologic confirmation of their diagnoses by biopsy or Whipple resection, while 32 were shown to have adenocarcinoma by radiology (computed tomography scan, magnetic resonance imaging, arteriogram, or endoscopic ultrasound). In the latter group, 6 patients had their diagnoses confirmed by biopsy or Whipple resection, and 19 had them supported by radiology.

The 19 patients with atypical fine-needle aspirates were followed up carefully. Twelve of these patients were shown to have evidence of malignancy. Three of these 12 patients underwent a Whipple resection (pancreatoduodenectomy) for their disease. Of the 7 patients who had atypical aspirates and who never developed adenocarcinoma clinically, 4 underwent a resection or biopsy that showed either chronic pancreatitis (n=3) or a serous cystadenoma (n=1). Therefore, with clinical follow-up information added to the cytologic diagnoses, 63 patients (67%) in this study had adenocarcinoma of the pancreas, by cytology and/or clinical follow-up (48 with positive cytologic diagnoses, 3 with false-negative cytologic diagnoses, and 12 with atypical cytologic diagnoses who developed adenocarcinoma), and 31 (33%) did not have adenocarcinoma of the pancreas.

There were no significant differences in clinical characteristics among patients in each of the four cytologic groups (p=1.000 for age; p=0.193 for sex).

K-ras mutations

Twenty-eight (30%) of the 94 specimens harbored an activating point mutation at codon 12 of the K-ras gene. Only 3 of the 6 previously identified different activating mutations at codon 12 were seen among the 28 positive cases: 12 (43%) were GGT (Gly) to GTT (Val) mutations, 11 (39%) GGT to GAT (Asp) mutations, and 5 (18%) GGT to CGT (Arg) mutations. No activating point mutations to TGT (Cys), AGT (Ser), or GCT (Ala) were identified (figure 2).

The presence of a K-ras mutation was analyzed with respect to original cytologic diagnosis. Twenty-four (50%) of 48 samples diagnostic of adenocarcinoma harbored K-ras mutations, while 3 (16%) of 19 samples suggestive of adenocarcinoma contained K-ras mutations. In contrast,
only one negative cytologic sample harbored a K-ras mutation (GTT, Val). This negative sample with a K-ras mutation did not originate in one of the three patients with false-negative cytologic diagnoses. The two samples diagnosed as neoplasms other than adenocarcinoma did not have K-ras mutations (table 1). The differences in prevalence of K-ras mutations among all of these diagnostic categories were highly statistically significant (p ≤ 0.001).

The three patients with atypical cytologic samples that also had K-ras mutations were studied. Clinical follow-up showed that each of these three patients had adenocarcinoma of the pancreas. The cytologic samples from two of the patients also showed alterations in the p53 and DPC4 (MAD4) genes, and these two patients died 3 and 4 months after their initial cytological diagnoses. The patient whose cytology sample showed a K-ras mutation but no other molecular change was a 70-year-old man who was alive 23 months after his initial diagnosis. Of the 16 patients with atypical aspirates that did not show K-ras mutations, 9 had adenocarcinoma of the pancreas on clinical follow-up. Therefore, compared with cytologic evaluation alone, a positive K-ras test identified three additional patients who had pancreatic adenocarcinoma. The absence of a mutation (ie, a wild-type K-ras gene) did not, however, exclude the diagnosis of cancer. Thus, 27 (43%) of 63 patients with pancreatic cancer on cytology or follow-up had a positive K-ras test result, while 30 (97%) of 31 patients without pancreatic adenocarcinoma by cytology and

**Figure 2** An autoradiogram of K-ras mutational analysis in fine-needle aspirates of the pancreas. Seven nylon membranes, each hybridized with a different radioactively labeled oligonucleotide specific for the sequence of the wild-type codon 12 (left) and the 6 possible mutations. On each membrane in the left lane are the unenriched polymerase chain reaction (PCR) products, the right lane contains the mutant-enriched PCR products. A mutant specimen should create a weak signal in the unenriched and a strong signal in the enriched columns, because enrichment increases the proportion of mutant DNA. Row 1 contains the hybridization controls, DNA complementary to the labeled oligonucleotide. Rows 2 and 6 show samples that were negative for malignancy. Rows 3 and 5 contain samples diagnosed by cytology as suggestive, but not diagnostic, of malignancy. They harbor Gly (GGT) to Asp (GAT) and Val (GTT) mutations at codon 12 of the K-ras gene, respectively. Rows 4, 7, 8, and 9 represent samples that were diagnostic of adenocarcinoma. Rows 10 and 11 are positive controls showing 1 cell with mutant codon 12, coding for the amino acid valine, mixed in 100 and 1,000 cells with wild-type codon 12, respectively. Row 12 is a contamination control sample (water only). Row 13 is an amplification control sample (placenta DNA). Ala, alanine; Arg, arginine; Asp, aspartic acid; Cys, cysteine; Ser, serine; Val, valine; WT, wild-type = glycine.
follow-up had a negative K-ras test result. These numbers reflect the sensitivity and specificity of K-ras mutations for the diagnosis of pancreatic ductal adenocarcinoma in fine-needle aspirates, respectively (table 2, 3, and 4). The differences in prevalence of K-ras mutations between patients with and without final diagnoses of adenocarcinoma were highly statistically significant (p ≤ 0.001). Table 4 summarizes the test characteristics of the K-ras assay.

**Table 2** Correlation between final clinical - cytologic diagnosis and molecular diagnosis

<table>
<thead>
<tr>
<th>Final diagnosis</th>
<th>Mutant K-ras</th>
<th>PS3 labeling</th>
<th>Loss of DPC4 labeling</th>
<th>Entire panel, 1 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma (n = 63)</td>
<td>27 (43)</td>
<td>30 (48)</td>
<td>13 (21)</td>
<td>42 (67)</td>
</tr>
<tr>
<td>Negative for adenocarcinoma (n = 31)</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>2 (6)</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage).

**Table 3** Correlation between final clinical - cytologic diagnosis and number of positive molecular test results

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>21 (33)</td>
<td>24 (38)</td>
<td>8 (13)</td>
<td>10 (16)</td>
<td>63</td>
</tr>
<tr>
<td>Negative for adenocarcinoma</td>
<td>29 (94)</td>
<td>2 (6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>26</td>
<td>8</td>
<td>10</td>
<td>94</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage).

**Table 4** Test characteristics of cytology and molecular markers in all included fine-needle aspirates (n = 94)

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>76 (68.85)</td>
<td>81 (73.89)</td>
<td>89 (83.95)</td>
<td>63 (53.72)</td>
<td>3.94</td>
</tr>
<tr>
<td>43 (33.53)</td>
<td>97 (93.100)</td>
<td>96 (93.100)</td>
<td>45 (35.56)</td>
<td>13.29</td>
</tr>
<tr>
<td>48 (38.58)</td>
<td>97 (93-100)</td>
<td>97 (93-100)</td>
<td>48 (38.58)</td>
<td>14.76</td>
</tr>
<tr>
<td>21 (12.29)</td>
<td>100</td>
<td>100</td>
<td>38 (28.48)</td>
<td>--</td>
</tr>
<tr>
<td>67 (57.76)</td>
<td>94 (89.99)</td>
<td>95 (91-100)</td>
<td>58 (48.68)</td>
<td>10.33</td>
</tr>
<tr>
<td>86 (79.93)</td>
<td>94 (89.99)</td>
<td>96 (93-100)</td>
<td>76 (68.85)</td>
<td>13.29</td>
</tr>
</tbody>
</table>

NPV, negative predictive value; PPV, positive predictive value. * Data are given as percentages except for the likelihood ratios. The 95% confidence intervals are given in parentheses. Prevalence is the proportion of patients with malignancy.

**P53 immunohistochemical analysis**

Of the 94 cases, 31 (33%) showed nuclear labeling for (figure 3). In 21 (68%) of these 31 cases, there were more than 50 cells showing nuclear labeling for p53. In 10 cases (32%), there were fewer than 50 positive cells within the cytology sample, with a range from 5 cells (2 cases) to 25 cells (1 case). The remaining cases contained 6 (2 cases), 10 (2 cases), 15 (1 case), 20 (1 case), and 24 (1 case) cells that labeled for p53.
Twenty-five (52%) of the 48 cases that were cytologically diagnostic of adenocarcinoma labeled, as did 5 (26%) of the 19 atypical samples. In contrast, only one (4%) of the 25 negative samples stained for p53. The negative sample containing aberrant p53 expression did not originate in one of the three patients with false-negative cytologic diagnoses. The two neoplasms that were not adenocarcinomas were negative for p53. Table 1 summarizes the results of p53 immunohistochemical analysis on the fine-needle aspiration samples. The differences in prevalence of p53 immunoreactivity among all of these diagnostic categories were highly statistically significant (p ≤ .001).

The five atypical cytologic samples that also had p53 protein expression were analyzed. The cytologic samples from two of the five patients also showed alterations in the K-ras and DPC4 (MAD4) genes. Clinical follow-up showed that each of the five patients with atypical cytology that harbored p53 overexpression had adenocarcinoma of the pancreas. Of the 14 patients with atypical aspirates that did not show p53 overexpression, 7 had adenocarcinoma of the pancreas on clinical follow-up. Therefore, compared with cytologic evaluation alone, a positive p53 test result identified five additional patients who had pancreatic adenocarcinoma. Again, the absence of a detected p53 abnormality could not be used to exclude the diagnosis of carcinoma.

Thus, 30 (48%) of 63 patients with pancreatic cancer on cytology or follow-up had a positive p53 test result, while 30 (97%) of 31 patients without pancreatic adenocarcinoma by cytology and follow-up had a negative p53 test result. These numbers reflect the sensitivity and specificity of p53 immunohistochemical analysis for the diagnosis of pancreatic ductal adenocarcinoma in fine-needle aspirates, respectively (tables 2-4). The differences in prevalence of p53 immunoreactivity between patients with and without a final diagnosis of adenocarcinoma were highly statistically significant (p ≤ .001). Table 4 summarizes the test characteristics of the p53 assay.
**DPC4 (MAD4) immunohistochemical analysis**

Of the 94 fine-needle aspirates, 13 (14%) showed loss of DPC4 (MAD4) expression (figure 4). Eleven (23%) of the 48 samples that were diagnostic of adenocarcinoma showed loss of DPC4 (MAD4) expression, as did two (11%) of the 19 that were suggestive of adenocarcinoma. None of the samples showing either no evidence for malignancy or a neoplasm other than adenocarcinoma showed loss of DPC4 (MAD4) (table 1). The differences in prevalence of loss of Dpc4 labeling among all of these diagnostic categories were statistically significant ($P = .020$).

The two atypical cytologic samples that also showed DPC4 (MAD4) loss were from the two patients whose cytology also had K-ras and p53 alterations. As discussed in the "K-ras mutations" section, these patients both had pancreatic adenocarcinomas; they died 3 and 4 months after their initial cytologic diagnoses. Of the 17 patients with atypical aspirates that did not show

**Figure 4** Examples of Dpc4 immunohistochemical assays. (A) This specimen read as negative for malignancy shows intact expression of the Dpc4 protein. (B) This aspiration was diagnostic of adenocarcinoma; Dpc4 expression is lost. (*400*) (page 257)

DPC4 (MAD4) loss, 10 had adenocarcinoma of the pancreas on clinical follow-up. Thus, compared with cytologic evaluation alone, a positive DPC4 (MAD4) test result (actually a loss of Dpc4 [Mad4] expression) identified two additional patients who had pancreatic adenocarcinoma. Therefore, 13 (21%) of 63 patients with pancreatic cancer on cytology or follow-up had a positive DPC4 (MAD4) test result, while 31 (100%) of 31 patients without pancreatic adenocarcinoma by cytology and follow-up had a negative DPC4 (MAD4) test result. These numbers reflect the sensitivity and specificity of DPC4 (MAD4) immunohistochemical analysis for the diagnosis of pancreatic ductal adenocarcinoma in fine-needle aspirates, respectively (tables 2 and 4). The differences in prevalence of loss of Dpc4 labeling between patients with and without a final diagnosis of adenocarcinoma were statistically significant ($p=0.004$). Table 4 summarizes the test characteristics of the DPC4 (MAD4) assay.
Summary of molecular and cytologic findings

Each of the cytologic samples was evaluated with respect to the entire panel of molecular markers. Of the 48 aspirates cytologically diagnosed as diagnostic for malignancy, 12 showed no molecular alterations, 20 showed one molecular alteration, eight showed two molecular alterations, and eight showed three molecular alterations (i.e. 36/48 samples (75%) showed at least one molecular alteration). Of the 19 aspirates that were suggestive of malignancy, 13 showed no molecular alterations, four showed one molecular alteration, none showed two molecular alterations, and two showed three molecular alterations. Twenty-three (92%) of the 25 negative aspirates contained no molecular alterations; two of these samples, however, harbored one molecular alteration each. Neither of these samples with a molecular alteration came from any of the three patients with false-negative cytologic diagnoses. Both of the samples recorded as diagnostic of neoplasm other than adenocarcinoma were negative for all of the molecular markers.

The 19 patients with atypical cytologic diagnoses were studied in more detail. Of the 13 patients with cytologic samples showing no molecular alterations, six had pancreatic adenocarcinoma on follow-up. Five of these six patients died, with an average survival time of 8.0 months; the remaining patient was alive 14 months after cytologic diagnosis. Of the six patients with at least one molecular alteration, all six had pancreatic adenocarcinoma. As discussed in the “K-ras mutations” and “p53 Immunohistochemical Analysis” sections, three of four patients with one molecular alteration had p53 overexpression in their aspirates, while one had a K-ras mutation. The other two had alterations in each of the three molecular markers. Therefore, a panel of K-ras, p53, and DPC4(MAD4) molecular alterations identified six additional patients who had pancreatic adenocarcinoma but whose cytologic specimens were not diagnostic of adenocarcinoma. The test characteristics of the molecular markers in the subgroup of samples with “suspicious” cytologic findings are summarized in Table 5.

Thus, considering the entire panel of tests together, 42 (67%) of 63 patients with pancreatic cancer on cytology and/or follow-up had at least one positive molecular test result, while 29 (94%) of 31 patients without pancreatic adenocarcinoma by cytology and follow-up did not have any positive molecular test results. This difference in the presence of at least one positive molecular

Table 5 Test characteristics of molecular markers in the subgroup of samples with “suspicious” cytologic findings (n=19) *

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Positive</th>
<th>Negative</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-ras</td>
<td>25 (6-44)</td>
<td>100</td>
<td>100</td>
<td>44 (21-66)</td>
<td>-</td>
<td>-</td>
<td>63 (41-85)</td>
</tr>
<tr>
<td>p53</td>
<td>42 (19-64)</td>
<td>100</td>
<td>100</td>
<td>50 (28-72)</td>
<td>-</td>
<td>-</td>
<td>63 (41-85)</td>
</tr>
<tr>
<td>DPC4(MAD4)</td>
<td>17 (0-33)</td>
<td>100</td>
<td>100</td>
<td>41 (19-63)</td>
<td>-</td>
<td>-</td>
<td>63 (41-85)</td>
</tr>
<tr>
<td>Entire panel, 1 mutation</td>
<td>50 (28-72)</td>
<td>100</td>
<td>100</td>
<td>54 (31-76)</td>
<td>-</td>
<td>-</td>
<td>63 (41-85)</td>
</tr>
</tbody>
</table>

NPV, negative predictive value; PPV, positive predictive value.

* Data are given as percentages. The 95% confidence intervals are given in parentheses. Prevalence is the proportion of patients with malignancy.
test was highly statistically significant between these two groups ($p \leq 0.001$). These numbers also reflect the sensitivity and specificity of at least one positive molecular test result in diagnosing pancreatic ductal adenocarcinoma in fine-needle aspirates, respectively (tables 2-4). Table 4 summarizes the test characteristics of the three molecular assays.

**DISCUSSION**

Fine-needle aspiration is a useful technique in the diagnosis of pancreatic ductal adenocarcinoma. While most aspirates are diagnostic of a malignant or benign condition, a substantial minority is atypical and cannot adequately rule in or rule out the presence of an adenocarcinoma. If ancillary techniques could help determine which of these atypical biopsy specimens were in actuality benign or malignant, patients would be saved additional procedures, which could result in decreased morbidity and mortality.

In the present study, we took advantage of the fact that pancreatic ductal adenocarcinoma is a genetic disease. Because most adenocarcinomas harbor alterations in the K-ras, $p53$, and $DPC4$ (MAD4) genes, the presence of such alterations could suggest the presence of an adenocarcinoma, even if a cytologic sample were only suggestive of malignancy. If molecular markers could identify or rule out malignancy in patients who did or did not have adenocarcinomas of the pancreas, additional tests would not be needed. The technique chosen for each of the molecular markers in this study—a dot-blot assay for the K-ras gene and immunohistochemical analysis for the $p53$ and $DPC4$ (MAD4) proteins—was easy to perform and interpret.

Molecular techniques already have been used on various samples, including cytologic ones, in making definitive diagnoses. For example, it can be very difficult to distinguish a benign bile duct proliferation, such as a bile duct adenoma, from a well-differentiated pancreatic adenocarcinoma metastasis in the liver on histologic grounds alone. However, virtually every liver metastasis of ductal adenocarcinoma harbors an activating point mutation in K-ras, but only a minority of benign bile duct proliferations do. Therefore, the presence of a K-ras mutation can help establish the diagnosis of pancreatic ductal adenocarcinoma metastatic to the liver.

In addition, several groups have used K-ras and $p53$ mutational status to help in the diagnosis on cytologic specimens. However, no group has yet used a panel of molecular changes that has included the $DPC4$ (MAD4) gene to assess malignancy on cytologic specimens. This is an important omission because, unlike alterations in the K-ras and $p53$ genes, $DPC4$ (MAD4) mutations and deletions are relatively specific for pancreatic adenocarcinoma.

In the present study, we showed that a panel of molecular-based tests can correlate with and supplement cytologic diagnosis in fine-needle aspirates of the pancreas. Seventy-five percent of all fine-needle aspirates diagnostic of adenocarcinoma had at least one molecular alteration, while 23 (92%) of 25 fine-needle aspirates read as negative for malignancy contained none of
the molecular alterations assayed in this study. In addition, in 6 of 12 aspirates diagnosed as atypical that came from patients who had evidence of a malignancy, at least one positive molecular marker was identified. None of the patients who had atypical aspirates but did not have evidence of malignancy on follow-up had a positive molecular marker.

With respect to the three genes in this study, alterations in the p53 and K-ras genes seem to be more sensitive for detecting adenocarcinoma within fine-needle aspirates of the pancreas. Close to half of the cases diagnostic for adenocarcinoma contained a p53 or a K-ras alteration each. In contrast, only one fifth of the cases diagnostic of adenocarcinoma showed loss of the DPC4 (MAD4) protein.

The sensitivities for all three of these genes, however, are lower than expected, given the known rates of molecular alterations in pancreatic adenocarcinomas. The lower rates produced in this study are most likely due to sampling error. For example, nonneoplastic cells on the p53 or DPC4 (MAD4) immunohistochemical slides may have obscured the labeling in rare cancer cells and, thereby, may have resulted in a false-negative result. This is especially true with DPC4 (MAD4) labeling, in which only one admixed cell that labeled for DPC4 (MAD4) could obscure the loss of labeling in other cells.

With respect to specificity, the DPC4 (MAD4) assay is better than the K-ras or p53 assays. While the latter tests were each positive on one sample that was negative for malignancy, there was never a loss of the DPC4 (MAD4) protein in a sample clinically diagnosed as negative for adenocarcinoma. These results are in keeping with the findings of previous molecular studies, which have shown that DPC4 (MAD4) loss is never present in benign pancreatic tissue.\(^{24,25}\) Therefore, the strength of the DPC4 (MAD4) labeling technique lies in its specificity, which is an important test characteristic for clinical practice.

One problem with this study is the low number of patients who had histologic confirmation of their cancers. It is not in dispute that these patients indeed had cancer, because radiologic and clinical follow-up showed progression of disease in these cases. However, it is possible that these patients had cancers other than pancreatic ductal adenocarcinomas, for example, other primary pancreatic neoplasms, periampullary carcinomas, lymphomas, or metastases. These cancers are capable of mimicking pancreatic ductal adenocarcinoma both clinically and radiologically, and these neoplasms may have different molecular profiles from pancreatic adenocarcinoma. This may contribute to the low sensitivity of our molecular panel in that we assumed that all patients with the clinical and radiologic appearance of pancreatic cancer had pancreatic ductal adenocarcinoma. It is important to note, however, that a screening test for pancreatic cancer would be most valuable if it could identify patients with any type of cancer involving the pancreas, since the Whipple procedure is the treatment for all of these neoplasms. It is therefore valid, and indeed preferable, to include all patients with cancer in the pancreas and periampullary region,
whether pancreatic ductal adenocarcinoma or not, in calculating test characteristics for the molecular panel used in this study.

Another problem with this study is the use of radiology as a surrogate for histologic confirmation, even in patients without cancer and even with only a 16-month follow-up. We believe that this is not particularly troublesome because the overwhelming majority of patients with pancreatic ductal adenocarcinoma will have had progression of their disease by 16 months of follow-up. Thus, patients who have pancreatic ductal adenocarcinoma but who have a false-negative cytologic diagnosis most likely would have evidence of disease progression by 16 months of follow-up time. Therefore, radiology is a valid way to support the diagnosis of benignancy (or at least "negativity for adenocarcinoma") in this particular situation. Indeed, it is "negativity for (ductal) adenocarcinoma" that is important in this study, since we presume that the molecular panel used in this study is targeted toward patients with ductal adenocarcinoma of the pancreas. Therefore, taken together, a panel of molecular markers is an excellent way to identify some patients who have adenocarcinoma of the pancreas but whose cytologic specimens are not diagnostic of malignancy. Each of the molecular tests in this panel is easy to perform and interpret. A molecular panel may clarify a diagnosis in patients who have atypical aspirates, and its use may lead to earlier and less invasive diagnosis. Therefore, a panel of molecular markers may be an excellent supplement to traditional cytology.


