Clinical significance of molecular markers in pancreatic cancer
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Immunohistochemical Labeling for the DPC4 Gene Product is a Specific Marker for Adenocarcinoma in Biopsy Specimens of the Pancreas and Bile Duct

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Abstract

We immunohistochemically labeled 72 biopsy specimens from the extrahepatic biliary tree and pancreas for Dpc4 protein and correlated expression with histologic diagnosis and patient follow-up. Specimens were classified histologically as follows: nonneoplastic, 35; neoplastic, 22; atypical, 15. Loss of expression of Dpc4 protein was identified in 12 specimens; 11 were histologically diagnostic of carcinoma. The 12th specimen was from a patient whose biopsy specimen initially was diagnosed as "atypical," but clinical follow-up revealed adenocarcinoma. Of the 12 atypical biopsy specimens with intact expression for Dpc4, follow-up later revealed that 10 were adenocarcinoma. Loss of expression of Dpc4 protein was never identified in a benign specimen.

Immunohistochemical labeling for the Dpc4 gene product is a specific marker of carcinoma in biopsy specimens of the pancreas and extrahepatic bile ducts and is marginally helpful in classifying atypical specimens. The sensitivity for carcinoma is low. This latter finding is not unexpected, because the DPC4 tumor suppressor gene is inactivated in only about half of pancreatic and biliary malignant neoplasms. Importantly, loss of Dpc4 expression has been reported in in situ carcinomas, suggesting that loss of expression should not be equated with invasive carcinoma.

The DPC4 gene is inactivated in approximately 55% of pancreatic ductal adenocarcinomas and in 15% to 55% of extrahepatic biliary adenocarcinomas.1-5 Inactivation occurs by 1 of 2 mechanisms: (1) intragenic mutation in one allele coupled with loss of the other allele (loss of heterozygosity) or (2) deletion in both alleles (homozygous deletion).6 Homozygous deletion inactivates the gene in 35% of pancreatic adenocarcinomas, and intragenic mutation coupled with loss of the second allele inactivates it in another 20%.1-5 Compared with other genetic changes that occur in pancreas cancer, inactivation of the DPC4 gene is relatively specific for the disease.1-11

Unfortunately, searching primary tumor tissue genetically for deletions can be difficult, especially in a neoplasm like pancreatic adenocarcinoma. This is because pancreatic adenocarcinomas evoke intense desmoplastic responses, and, as a result, most of the DNA obtained from tumor samples is actually nonneoplastic DNA. Immunohistochemical analysis can overcome this problem because it is performed in situ, and the histologic features of the tissue are preserved. In addition, it can be applied to formalin-fixed tissues processed in a routine manner. Indeed, Wilentz et al12 have recently shown that immunohistochemical analysis for the Dpc4 protein performed on pancreatic resection specimens is an extremely sensitive and specific technique to classify DPC4 gene status.

Although immunohistochemical analysis is an accurate way to identify inactivation of the DPC4 gene, this technique has not been validated as a diagnostic tool. One excellent way to test this technique's usefulness is to study it in a set of biopsy specimens from the pancreas and extrahepatic bile ducts from patients with detailed clinical follow-up. This
study would help determine whether this technique could be used diagnostically in small tissue samples and could establish a malignant diagnosis in specimens suggestive but not diagnostic of carcinoma by examination of histologic features alone. Patient follow-up would be used as the “gold standard” to verify all patients’ diagnoses.

If Dpc4 immunohistochemical analysis could help establish malignant diagnoses in even a fraction of the biopsy specimens not quite diagnostic of carcinoma by histologic features alone, patients would benefit greatly. This is so because in these cases, additional diagnostic procedures, such as repeated imaging, further biopsies, endoscopic ultrasound, and even laparotomy, often are necessary to diagnose a carcinoma. The morbidity and mortality attending additional diagnostic procedures could be avoided if the use of immunohistochemical analysis could establish a diagnosis.

### Materials and Methods

#### Specimen Selection

We included 72 biopsy specimens from the pancreas and extrahepatic bile ducts, obtained from 63 patients, in the study. These specimens represented cases of pancreatic and biliary tract biopsies performed between September 1996 and February 1999 at the Johns Hopkins Hospital, Baltimore, MD. Although 125 biopsy specimens originally were available for study, the paraffin blocks from 53 biopsy specimens produced slides that failed to react with the antibody (n = 11) or that did not contain enough tissue to attempt immunohistochemical analysis (n = 42).

The 72 specimens were obtained from patients undergoing endoscopic retrograde cholangiopancreatography, open laparotomy, or radiologically guided percutaneous biopsy. Thirty-six were from the pancreas, and 36 were from the extrahepatic biliary tree. Of the latter group, 18 were from the distal common bile duct, 9 were from the proximal common bile duct, and 9 were from the common, right, or left hepatic ducts. Thirteen patients had resections of their pancreatic or biliary masses after biopsy (10 Whipple resections, 3 distal pancreatectomies). Tissue blocks of tumor from 6 of these resection specimens were available for study.

#### Data Procurement

Clinical and pathologic data for the 72 cases were obtained from patients’ medical records, the Johns Hopkins Oncology Center information system database, and the Johns Hopkins Hospital Surgical Pathology files. Clinical and pathologic characteristics studied included sex, race, and age.

### Immunohistochemical Analysis

Unstained 4-μm sections were cut from the paraflin block of each case and deparaffinized by routine techniques. The slides were treated with sodium citrate buffer (diluted to 1x from 10x heat-induced epitope retrieval buffer, Ventana BioTek Solutions, Tucson, AZ) and then steamed for 20 minutes at 80°C. After cooling for 5 minutes, the slides were labeled with monoclonal antibody to Dpc4 (clone B8, Santa Cruz Biotechnology, Santa Cruz, CA) using the BioTek-Mate 1000 automated stainer (Ventana BioTek Solutions). Each slide was labeled with a 1:100 dilution of the antibody. The anti-Dpc4 antibody was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3,3’-diaminobenzidine. Sections were counterstained with hematoxylin.

Two of us (M.T., R.E.W.) simultaneously evaluated the immunohistochemical labeling of the biopsy specimens. We were unaware of the previously made diagnosis on the originally cut H&E-stained slides for each biopsy specimen. The labeling in each case was scored as positive or negative. Positive labeling was defined as strong and uniform expression of Dpc4 in the cytoplasm of cells, with at least focal expression of Dpc4 in nuclei. Cases were regarded as negative only when no expression of Dpc4 was seen in the cytoplasmic or nuclear compartments of cells. In the 6 resection specimens studied, the staining category of “focally positive” also was used when a tumor contained 2 distinct populations of cells, those that labeled with the antibody to Dpc4 and those that did not. The interpretation of immunohistochemical labeling was highly reproducible, with agreement between both observers in all cases.

Normal ductal epithelium, islets of Langerhans, pancreatic acini, lymphocytes, and stromal fibroblasts, which uniformly show moderate to strong expression of the Dpc4 gene product, served as positive internal controls in each of the sections.

### Statistical Analysis

Cross tabulations were analyzed with chi-square tests. Means were compared with the Wilcoxon signed rank test. Each test was 2-tailed.

### Results

#### Histologic Diagnoses on the Biopsy Specimens

We included 72 biopsy specimens from the pancreas and extrahepatic biliary tree in the study. Thirty-six specimens originated in the pancreata of 33 patients Imagen 18. Seventeen biopsy specimens were diagnosed as a neoplasm (15 adenocarcinomas, 1 adenosquamous carcinoma, and 1
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**Image 11** Pancreatic biopsy specimen. A. Malignant glands infiltrating stroma (H&E, x160). B. An immunohistochemical stain against the Dpc4 protein is negative (x160). Intervening nonneoplastic ducts served as a positive internal control.

Islet cell tumor. Fourteen were interpreted as nonneoplastic. Finally, 5 of the specimens were suggestive but not diagnostic of carcinoma. These 5 atypical biopsy specimens were from 5 different patients.

Thirty-six biopsy specimens from 30 patients originated in the extrahepatic biliary tree. Of these, 18 were from the distal common bile duct, 9 were from the proximal common bile duct, and 9 were from the common, right, or left hepatic ducts. Of the 18 distal common bile duct specimens, 4 were diagnostic of adenocarcinoma, 10 were nonneoplastic, and 4 were atypical. **Image 21, Image 31.**

Four of the proximal common bile duct biopsy specimens were nonneoplastic, and 5 were atypical. There were 7 nonneoplastic hepatic duct specimens; 1 of the hepatic duct specimens was diagnosed as adenocarcinoma, and 1 was suggestive of adenocarcinoma. Therefore, among the extra-hepatic biliary tract biopsy specimens, 5 contained adenocarcinoma, 21 showed no evidence of malignancy, and 10 were atypical. The 10 atypical biopsy specimens were from 7 different patients.

**Image 21** Distal common bile duct biopsy specimen that was suggestive but not diagnostic of adenocarcinoma (A), but it showed loss of Dpc4 expression (B, arrow), supporting a diagnosis of adenocarcinoma. Entrapped nonneoplastic gland formed a positive internal control (arrowhead). (H&E, x160)
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Patient Diagnoses After Follow-up

Each of the 63 patients was followed up clinically after his or her original biopsy diagnosis. For the 22 patients who had at least 1 biopsy specimen diagnostic of a neoplasm, diagnoses were confirmed by clinical and radiologic follow-up (n = 19) or histologic examination of a resection specimen (n = 3). Of the 30 patients without evidence of malignancy on any of their 33 biopsy specimens, 11 subsequently were proved to have a malignant neoplasm, most likely missed by sampling error in the biopsy. These malignant neoplasms were verified by clinical follow-up (n = 7) or by histologic examination of a subsequent resection specimen (n = 4). One of these malignant neoplasms actually was renal cell carcinoma metastatic to the pancreas, confirmed on examination of a Whipple specimen.

The 12 patients with biopsies interpreted as “atypical” also were carefully followed up. Two of these patients had no evidence of cancer, even after close clinical follow-up for 45 months (n = 1) or a Whipple resection (n = 1). However, 10 of the patients did have cancer, diagnosed by clinical follow-up (n = 6), histologic examination of a Whipple resection specimen (n = 3), or a synchronous diagnostic biopsy (n = 1). Nine of the 10 cancers were primary carcinomas; 1 patient with a biopsy interpreted as atypical actually had a colon cancer metastatic to the distal common bile duct. One of the 9 primary carcinomas was an invasive adenocarcinoma that arose in association with an intraductal papillary mucinous neoplasm. Finally, 1 patient had 4 separate biopsy specimens suggestive but not diagnostic of cancer; this patient was 1 of the 6 found to have adenocarcinoma on clinical follow-up.

In summary, 42 patients had evidence of a neoplasm. In all cases but 3 (islet cell tumor, metastatic colon cancer, metastatic renal cell carcinoma), the neoplasm was a primary pancreatic or biliary adenocarcinoma or variant (adenosquamous carcinoma). However, for 11 patients with a malignant neoplasm, the cancer was missed by biopsy, and another 9 biopsy specimens were interpreted as atypical, never accompanied by a biopsy diagnostic of cancer. Twenty-one patients had no definitive evidence of malignant neoplasm revealed by biopsy, clinical follow-up, or examination of a subsequent resection specimen.

Immunohistochemical Analysis

Twelve (17%) of 72 biopsy specimens included in the study showed loss of Dpc4 expression. Eleven (52%) of 21 biopsy specimens diagnostic of adenocarcinoma showed loss of Dpc4 expression. In addition, 1 (7%) of the 15 biopsy specimens suggestive but not diagnostic of adenocarcinoma was negative for Dpc4. None of the 35 nonneoplastic biopsy specimens showed loss of Dpc4 expression. Table 1 summarizes these immunohistochemical data.

All 21 patients with biopsy specimens diagnostic of adenocarcinoma were verified to have adenocarcinoma on follow-up. Therefore, using histologic features as the gold standard, the sensitivity of Dpc4 immunohistochemical analysis in identifying adenocarcinoma on biopsy was 52%. Since none of the benign biopsy specimens in the study showed loss of Dpc4 expression, the specificity of the technique was 100%.

The atypical biopsy specimen showing loss of Dpc4 expression originally was diagnosed as atypical because
significant crush artifact precluded a definitive diagnosis of cancer (Image 2). The biopsy came from a 52-year-old Hispanic man who later was clinically shown to have adenocarcinoma of the distal common bile duct. This patient died 5 months after his biopsy. It is important to note that this patient did not have any simultaneous biopsy samples that were diagnostic of a malignancy. Therefore, immunohistochemical analysis helped establish a subsequent proven diagnosis of adenocarcinoma in 1 patient whose biopsy was not diagnostic of adenocarcinoma by histologic features alone.

**Immunohistochemical Analysis of Resection Specimens**

Tissue blocks from 1 distal pancreatectomy and 5 Whipple specimens performed after biopsies were taken were available for immunolabeling and comparison with the biopsy material. The Dpc4 status in slides from each resection specimen matched that in the corresponding original biopsy specimen. For example, the islet cell tumor seen both within the distal pancreatectomy and the corresponding original biopsy specimen expressed the Dpc4 protein. Two adenocarcinomas of the distal common bile duct with intact Dpc4 expression were identified within Whipple resections that were performed after benign diagnoses were made on biopsy; these benign biopsy specimens of course showed intact Dpc4 expression.

Three Whipple resections completed after the identification of atypical cells in biopsy specimens also were concordant with their corresponding Dpc4-positive biopsy specimens. In 2 of these Whipple resection specimens, both areas of in situ and infiltrating adenocarcinoma were diffusely positive with the Dpc4 stain. In the other resection specimen, the invasive adenocarcinoma of the pancreas was only focally positive, with some areas showing intact expression and other areas showing loss of Dpc4 expression.

**Clinical Characteristics**

The clinical characteristics of patients with malignant biopsy specimens that showed intact Dpc4 expression, malignant biopsy specimens that showed loss of Dpc4 expression, atypical biopsy specimens, and benign biopsy specimens were compared. These characteristics are summarized in Table 21. There were no significant differences among these 4 groups with respect to age ($P = 1.000$, Wilcoxon signed rank test), sex ($P = .205$, chi-square test), or race ($P = .338$, chi-square test).

**Discussion**

Approximately half of the adenocarcinomas of the pancreas and extrahepatic biliary tree have inactivated \( DPC4 \) genes. \(^5,6\) Inactivation occurs by deletion of both \( DPC4 \)
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alleles (homozygous deletion) or by mutation in one allele combined with loss of the other allele (loss of heterozygosity). Inactivation of the DPC4 gene is important in these cancers because compared with alterations in other genes, such as K-ras, p16, and p53, changes in DPC4 are relatively specific for pancreatic adenocarcinoma.1-3,7-10,14

The genetic analysis of the DNA from an infiltrating cancer for DPC4 alterations can be greatly limited by low neoplastic cellularity. Because it is technically less cumbersome and more widely available than are genetic assays, immunohistochemical analysis of the gene product is an excellent way to avoid this problem. Indeed, in a recent study, Wilentz et al12 found that immunohistochemical analysis for the Dpc4 gene product is a highly specific and sensitive marker for DPC4 gene inactivation.

However, even though the technique is highly sensitive and specific, the role of Dpc4 immunohistochemical analysis in clinical diagnosis has yet to be proven. We undertook this project to determine whether this technique is valuable diagnostically by studying it in a frequently encountered difficult clinical setting. By studying Dpc4 immunohistochemical analysis in benign, malignant, and atypical biopsy specimens of the pancreas and extrahepatic biliary tree, we hoped to show that Dpc4 immunohistochemical analysis can be a valuable adjunct to routine histologic examination in establishing the diagnosis of cancer in small tissue samples, especially those suggestive but not diagnostic of carcinoma by histologic examination alone.

Indeed, we showed in this study that immunohistochemical labeling for Dpc4 is a valuable technique in the diagnosis of adenocarcinoma in biopsy specimens from the pancreas and extrahepatic biliary tree. Importantly, the technique is 100% specific. In contrast, immunohistochemical analysis is only 52% sensitive. This low sensitivity is not surprising, given that only half of all pancreatic and biliary adenocarcinomas actually inactivate the DPC4 gene. More important, however, is that immunohistochemical analysis for Dpc4 successfully established the diagnosis of adenocarcinoma in a patient whose distal common bile duct biopsy specimen was suggestive but not diagnostic of adenocarcinoma by histologic examination alone. The presence of adenocarcinoma in this patient was verified by clinical follow-up.

Therefore, this study leads to two important conclusions about Dpc4 immunohistochemical analysis in biopsy specimens from the pancreas and biliary tract. First, the technique is a specific marker for adenocarcinoma of the pancreas and biliary tract. Thus, loss of Dpc4 expression in a biopsy of the pancreas or biliary tract helps establish the diagnosis of cancer. Furthermore, because Dpc4 inactivation is relatively specific for periampullary cancer, loss of Dpc4 expression signals the probability that the cancer is primary to the pancreas or biliary tree. It is important to note, however, that because biopsy specimens represent only part of a lesion, sampling error may be an issue. For example, atypical biliary or pancreatic epithelium showing loss of Dpc4 on biopsy may originate in a lesion showing focal positivity for Dpc4, seen only when the entire lesion is examined. This would mean that the lesion could not be definitively diagnosed as malignant, even though it may indeed be malignant (as approximately half of all pancreatic and biliary carcinomas stain for Dpc4 and as a minority of these do so in a focal pattern13). It is just as important to note, though, that in our experience, we have never identified a benign lesion that was even focally negative for Dpc4, although the possibility that this occurs certainly exists.

Second, this technique may be a valuable adjunct to histologic examination for establishing the diagnosis of cancer in patients with biopsy specimens that are only suggestive of cancer by histologic examination. In the present study, one patient's biopsy specimen that was suggestive but not diagnostic of adenocarcinoma showed loss of Dpc4 expression. Because loss of Dpc4 expression was never found in benign biopsy specimens, Dpc4 immunohistochemical analysis helped establish the definitive diagnosis of cancer in this case. It is important to note, however, that while Dpc4 loss may establish the diagnosis of cancer, it does not necessarily imply invasive adenocarcinoma. Wilentz et al15 have previously shown that Dpc4 loss occurs in a subset of in situ carcinomas of the pancreas. However, the clinical importance of this distinction may be nil, since the appropriate treatment for any localized invasive or in situ tumor would be the same, namely resection.

Because of its simplicity and availability, immunohistochemical labeling for Dpc4 in biopsy specimens from the pancreas and extrahepatic biliary tree has direct clinical applications. For example, labeling for Dpc4 may help to distinguish trapped glands in chronic pancreatitis (which should express Dpc4) from infiltrating adenocarcinomas of the pancreas (half of which will not express Dpc4). The use of this immunohistochemical technique may lead to quicker and more accurate diagnosis of cancers of the pancreas and extrahepatic biliary tree. Although the improvement over routine histologic examination is only marginal, the use of immunohistochemical analysis for Dpc4 may, nevertheless, be of value, because patients may be spared additional diagnostic procedures and the morbidity and mortality that accompany these procedures.

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