Short Communication

Immunohistochemical Labeling for Dpc4 Mirrors Genetic Status in Pancreatic Adenocarcinomas

A New Marker of DPC4 Inactivation

Robb E. Wilentz,* Gloria H. Su,*† Jia Le Dai,*†
Andrew B. Sparks,† Pedram Argani,*
Taylor A. Sohn,† Charles J. Yeo,†‡
Scott E. Kern,*† and Ralph H. Hruban*†

From the Departments of Pathology,* Oncology,† and Surgery,‡
The Johns Hopkins Medical Institutions, Baltimore, Maryland

DPC4 (MADH4, SMAD4) is a tumor suppressor gene inactivated by allelic loss in approximately 55% of pancreatic adenocarcinomas. Unfortunately, it can be technically very difficult to detect the inactivation of **DPC4** at the genetic level because genetic analyses require the microdissection of relatively pure samples of neoplastic and normal tissues. This is especially true for pancreatic adenocarcinomas, which elicit vigorous, non-neoplastic, stromal responses. Immunohistochemical labeling can overcome this hurdle because it preserves morphological information. We therefore studied the expression of the **DPC4** gene product in 46 cancers, including 5 cancer cell lines by Western blot analysis and 41 primary periampullary adenocarcinomas by immunohistochemistry. The status of exons 1–11 of the **DPC4** gene in all 46 of the cancers had been previously characterized at the molecular level, allowing us to correlate **Dpc4** expression directly with gene status. Three cell lines had wild-type **DPC4** genes, and **Dpc4** expression was detected in all three by Western blot. The two cell lines with homozygously deleted **DPC4** genes did not show **Dpc4** protein by Western blot analysis. Immunohistochemical labeling revealed that 17 (94%) of the 18 primary adenocarcinomas with wild-type **DPC4** genes expressed the **DPC4** gene product, whereas 21 (91%) of 23 primary adenocarcinomas with inactivated **DPC4** genes did not. Cases in which there was discordance between the immunohistochemical labeling and the genetic analyses were reanalyzed genetically, and we identified a deletion in exon 0 of **DPC4** in one of these cases. This is the first

report of a mutation in exon 0 of **DPC4** in a pancreatic cancer. The contrast between the strong expression of **Dpc4** by normal tissues and the loss of expression in the carcinomas was highlighted in several cases in which an infiltrating cancer was identified growing into a benign duct. These observations suggest that immunohistochemical labeling for the **DPC4** gene product is an extremely sensitive and specific marker for **DPC4** gene alterations in pancreatic carcinomas. The sensitivity and specificity of immunohistochemical labeling for **Dpc4** in other periampullary carcinomas has yet to be determined. (*Am J Pathol* 2000, 156:37–43)

DPC4 inactivation is one of the more common genetic alterations identified in pancreatic ductal adenocarcinomas.^{1–4} The **DPC4** tumor-suppressor gene is inactivated in these cancers by one of two mechanisms: 1) intragenic mutation in one allele coupled with loss of the other allele (loss of heterozygosity) or 2) deletion of both alleles (homozygous deletion).⁵ Indeed, the mapping of a consensus region of homozygous deletions in a series of pancreatic carcinomas first led to the discovery of the **DPC4** gene.^{1–3} Subsequent studies have shown that homozygous deletion inactivates **DPC4** in 35% of pancreatic adenocarcinomas and that intragenic mutation coupled with loss of the second allele inactivates **DPC4** in another 20%.^{1–3} Inactivation of the **DPC4** tumor suppressor gene is relatively specific for pancreatic cancer, although it does occur in a minority of cancers from other organs, including the colon, breast, ovary, and biliary

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Address reprint requests to Ralph H. Hruban, M.D., Meyer 7–181, Department of Pathology, The Johns Hopkins Hospital, 600 N. Wolfe Street, Baltimore, MD 21287. E-mail: rhruban@jhmi.edu.

tract.^{1-3,6-10} In addition, germline mutations in the *DPC4* gene have been implicated in juvenile polyposis.^{11,12}

Unfortunately, searching primary tumor tissue genetically for deletions can be difficult, especially in a neoplasm-like pancreatic adenocarcinoma. Pancreatic adenocarcinomas evoke intense desmoplastic responses, and, as a result, most of the DNA contained in a tumor is actually non-neoplastic DNA. This intimate admixture of normal and neoplastic tissues can mask the loss of a *DPC4* allele in a cancer. This problem can be overcome by expanding the tumor in culture or by xenografting the cancer into mice, but such research techniques are clearly not practical in most cases, and because they require fresh, sterile tissue, they cannot be done retrospectively.¹³

Immunohistochemical analysis can overcome the problem of low neoplastic cellularity, because it is performed *in situ*. Immunohistochemical analysis has three additional advantages over genetic analysis: 1) because tissue morphology is maintained, it allows a direct correlation between gene expression and morphology; 2) it is less labor intensive than molecular analyses, and, therefore, larger numbers of lesions can be examined; and 3) it can be applied to archived, formalin-fixed tissues processed in a routine fashion.

We therefore labeled 46 previously genetically well-characterized periaampullary adenocarcinomas with an antibody to the *DPC4* gene product. By comparing Western blotting or immunohistochemical labeling of the *DPC4* gene product with molecular genetic analyses of these same cancers, we could determine the specificity and sensitivity of this assay for the *DPC4* gene product. If immunohistochemistry for the Dpc4 protein were a sensitive and specific marker for *DPC4* genetic inactivation, then immunohistochemical analyses would be valuable in both investigative and clinical settings.

Materials and Methods

Specimen Selection and Data Procurement

Included in this study were 5 cancer cell lines and 41 cancer xenografts. The cancer lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Of the cell lines, two were pancreatic (BxPC3, PANC1), two were colorectal (HCT116, RKO), and one was mammary (MDA-MB-468) in origin. Of the xenografts, 36 were infiltrating ductal adenocarcinomas of the pancreas, two were adenocarcinomas arising in association with intraductal papillary mucinous neoplasms of the pancreas, two were adenocarcinomas of the distal common bile duct, and one was an (ampullary) adenocarcinoma of the region of the papilla of Vater. Each of these xenografted tumors was originally obtained from a patient undergoing either Whipple resection or distal pancreatectomy at The Johns Hopkins Hospital between May 4, 1992, and March 27, 1995, as previously described.¹³ The presence and sequence of exons 1-11 of the *DPC4* gene in each of these 46 cases had been determined in a previous study.^{1,2}

Clinical and pathological data for the xenografted cancers were obtained from patients' medical records, The Johns Hopkins Oncology Center information system database, and The Johns Hopkins Hospital Surgical Pathology files. Clinical and pathological characteristics included were gender, race, age, family history of cancer, alcohol use, tobacco use, symptoms, comorbidities, tumor differentiation, tumor size, tumor location, presence of nodal metastases at surgery, *K-ras* gene status, and microsatellite instability. Symptoms included jaundice, abdominal pain, weight loss, nausea/vomiting, and fevers/chills. Comorbidities included myocardial infarction, hypertension, peripheral vascular disease, diabetes mellitus, chronic obstructive pulmonary disease, peptic ulcer disease, acute pancreatitis, chronic pancreatitis, and inflammatory bowel disease.

Western Blot Analysis

Total cellular protein was harvested in Laemmli buffer without β -mercaptoethanol, and protein concentrations were determined by use of bicinchoninic acid reagents (Pierce, Rockford, IL). Fifty μ g of total protein were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by monoclonal antibody against Dpc4 (clone B8; working dilution, 4 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce). Detection was afforded by SuperSignal substrates (Pierce).

Immunohistochemistry

Multiple hematoxylin and eosin (H&E)-stained slides from each of the 41 primary cancers corresponding to the xenografted tumors were screened by light microscopy for sections having both infiltrating adenocarcinoma and non-neoplastic pancreas. Unstained 5- μ m sections were then cut from the paraffin block selected for each case and deparaffinized by routine techniques. The slides were treated with sodium citrate buffer (diluted to 1 $\times$ from 10 $\times$ heat-induced epitope retrieval buffer, Ventana-Bio Tek 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), using the Bio Tek-Mate 1000 automated stainer (Ventana). 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 the authors of this study (R. E. W. and R. H. H.) independently evaluated the immunohistochemical labeling of the carcinomas. Each author was unaware of the *DPC4* gene status of each case. The labeling in each case was scored as "diffusely positive," "focally positive," or "negative." "Diffusely positive" labeling was defined as strong and uniform expression of Dpc4 in the cytoplasm of cells, with focal expression of Dpc4 in nuclei. "Focally positive" carcinomas contained two distinct populations

of cells, those that labeled with the antibody to Dpc4 and those that did not. Cases were regarded as "negative" only when no expression of Dpc4 was seen in the cytoplasmic or nuclear compartments of cells.

The interpretation of immunohistochemical labeling of the cancers was highly robust, with agreement between the observers in all cases but one (PX68). One author scored the adenocarcinoma in this case as "focally positive," whereas the other scored it as "negative." After re-examination of this case by both authors, this case was considered to be "focally positive."

Cases known to show either homozygous deletion of the *DPC4* gene or a mutation combined with loss of heterozygosity in the *DPC4* gene were expected to show no expression of Dpc4. Cases known to have at least one wild-type *DPC4* gene were expected to show diffusely positive expression of Dpc4. When unexpected results were obtained, the immunohistochemical labeling was repeated with the same and/or new blocks. This included one *DPC4* homozygously deleted case designated as "diffusely positive," two *DPC4* wild-type cases designated as "focally positive," and one *DPC4* wild-type case designated as "negative." In each of these four cases,

repeat analysis confirmed the original immunohistochemical findings.

Normal pancreatic ducts, islets of Langerhans, acini, lymphocytes, and stromal fibroblasts, which all show moderate to strong expression of the *DPC4* gene product, served as positive internal controls in each of the sections.

Genetic Reanalysis of Cases Showing Unexpected Results by Immunohistochemistry

Each of the four cases showing unexpected results by immunohistochemistry was rescreened for homozygous deletions of exons 1 to 11 of the *DPC4* gene, as previously described.¹⁻³ In addition, a newly identified exon (exon 0) of the gene was also examined (B Vogelstein and AB Sparks, unpublished data). Exon 0 had not yet been identified when the original homozygous deletion screening of the 46 cancers was performed. Primers used for amplification of exon 0 by polymerase chain reaction were 5'-ACCTTCTCCCCAGAGCTGTGCG-3' and 5'-GAGCTCGGCGTAGAGTGGGCG-3'.

Statistical Analysis

Cross tabulations were analyzed with χ^2 or Fisher exact tests when appropriate. Means were compared with the Student's *t*-test or the nonparametric Mann-Whitney rank

Figure 1. Western immunoblot of five cancer cell lines. The three cancers expected to express Dpc4 (HCT116, RKO, and PANC1) contain 64-kD proteins recognizable by the anti-Dpc4 antibody. The two cell lines with homozygous deletions of *DPC4* (BxPC3 and MB468) do not express Dpc4. Dpc4 is a 64-kD protein.

Figure 2. Dpc4 expression in the normal pancreas. **A:** This normal pancreatic duct shows strong, diffuse cytoplasmic and occasional nuclear expression of Dpc4. Slightly weaker expression is seen in the surrounding stromal cells. **B:** The acini and islets of Langerhans (center) of the pancreas also express the Dpc4 protein. Note the regular nuclear labeling for Dpc4 in the islet.

Figure 3. **A:** An infiltrating adenocarcinoma with a wild-type *DPC4* gene expresses the Dpc4 protein. **B:** An infiltrating adenocarcinoma with a homozygously deleted *DPC4* gene (**bottom**) does not label with the anti-Dpc4 antibody. Note the strong labeling in the adjacent, non-neoplastic pancreatic acini (**top**). **C:** "Cancerization" of a duct, as demonstrated by Dpc4 immunolabeling. The normal cells within the duct express Dpc4 (**left**), whereas the infiltrating cancer cells growing into the duct do not express Dpc4 (**right**). Note the sharp contrast in Dpc4 expression between normal and cancer. **D:** An infiltrating carcinoma with two morphological patterns. The better differentiated, gland-forming adenocarcinoma (D1) expresses the Dpc4 protein, but the more poorly differentiated component (D2) does not.

sum test. The latter was used to compare distributions when the assumption of normality was not valid. All of these tests were two-tailed. Survival times between groups were calculated using the method of Kaplan and Meier¹⁴ and were compared using the log-rank statistic. Tests were performed using Statistica for Windows (Stat-Soft, Tulsa, OK).

Results

Western Blotting

The monoclonal antibody to Dpc4 was first tested by Western blotting. Dpc4 is a 64-kd protein. As seen in Figure 1, antibody to Dpc4 identifies a 64-kd product in the lysates of the HCT116, PANC1, and RKO cell lines. In contrast, no product is labeled within the lysates of BxPC3 and MDA-MB-468. These results match the previously known genetic characterizations of these cell lines.¹ HCT116, PANC1, and RKO all have wild-type *DPC4* genes, whereas the *DPC4* gene is homozygously deleted in BxPC3 and MDA-MB-468.^{1,3,9} Therefore, detection of Dpc4 by Western blot using the B8 monoclonal antibody directly mirrored the gene status.

Immunohistochemistry

In all cases, normal acinar, ductular, stromal, and islet cells were strongly labeled with the antibody (Figure 2). Both cytoplasmic and nuclear labeling was seen.

Figure 3 shows examples of immunohistochemical labeling of the cancers with antibody to the Dpc4 protein. Of the 41 periampullary adenocarcinomas, 17 (41%) were labeled diffusely positively; two (4.9%) were labeled focally positively; and 22 (54%) were not labeled. Sixteen (94%) of the 17 adenocarcinomas that were labeled diffusely positively had at least one wild-type *DPC4* allele (Figure 3A). The remaining carcinoma that was labeled diffusely positively in archival tissues had a homozygously deleted *DPC4* gene detected in xenografted tumor. The two adenocarcinomas that were labeled focally positively had at least one wild-type *DPC4* allele on genetic analysis. Of the 22 carcinomas that were not labeled with the antibody to the Dpc4 protein, 18 (82%) had homozygously deleted *DPC4* genes (Figure 3B), three (14%) had *DPC4* genes exhibiting an intragenic mutation of one allele and loss of heterozygosity in the other, and one (4.5%) had a wild-type *DPC4* allele. The three mutations included two nonsense mutations, TAC to

TAG (aa 412) and AGA to TGA (aa 515), and one deletion causing a frameshift, CAGAGCTCC to C (aa 516).

A striking finding identified by immunohistochemical labeling in some cases was the growth of an infiltrating adenocarcinoma into otherwise non-neoplastic ducts. In these cases the normal ducts strongly expressed the Dpc4 protein, whereas the infiltrating adenocarcinoma growing within the ducts did not (Figure 3C). This "cancerization of ducts" highlights the strong contrast between the labeling seen in the cancers and normal tissues.

One of the two carcinomas that labeled focally positively for Dpc4 had an interesting morphological pattern. The carcinoma from this case had two distinct morphologies. One was that of a moderately differentiated adenocarcinoma, and the other was a more poorly differentiated carcinoma that did not form glands. Remarkably, the gland-forming component was labeled with the anti-Dpc4 antibody, but the poorly differentiated component was not (Figure 3D). The other carcinoma that was labeled only focally for Dpc4 showed only the pattern of a moderately differentiated adenocarcinoma.

Genetic Reanalysis of Tumors Showing Unexpected Results by Immunohistochemistry

Four carcinomas showed unexpected results by immunohistochemistry. These included one *DPC4* homozygously deleted case designated as diffusely positive, two *DPC4* wild-type cases designated as focally positive, and one *DPC4* wild-type case designated as negative. Xenografts from each of these carcinomas were again screened for homozygous deletions, including a screen of a newly described *DPC4* exon 0 (exon 0) (B Vogelstein and AB Sparks, unpublished data).

One of the two carcinomas that originally were thought to have a wild-type gene but that expressed Dpc4 only focally was found to have a deletion in exon 0. The homozygous deletion was confirmed by using duplex polymerase chain reaction with a positive internal control primer set. Presumably the xenografted tissue for molecular analysis which showed the exon 0 deletion came from the poorly differentiated component of this neoplasm that did not label with the Dpc4 antibody (Figure 3D).

The *DPC4* gene status of the xenografts of the three other carcinomas (two wild type and one homozygously deleted) was verified with the repeat molecular assays. In particular, multiple parallel xenografts from one case were examined at the molecular level, and all parallel xenografts showed the same homozygous deletion, indicating origin of the homozygous deletion in the patient's primary carcinoma rather than during the establishment of the xenografts.

Summary of Results

Including the newly discovered exon 0 homozygous deletion, immunohistochemical labeling matched the genetic data in 38 (93%) of the 41 cases. Of 18 cases with wild-type *DPC4* genes, 17 (94%) were labeled diffusely or focally positively for the protein, and 21 (91%) of 23 cases

with inactivated *DPC4* genes were not labeled with the antibody. (See Table 1 for results.) Thus, immunohistochemistry for the *DPC4* gene product is a highly sensitive (91%) and specific (94%) marker for genetic alterations. Immunohistochemistry did not differentiate between a mutant *DPC4* allele combined with loss of heterozygosity and a homozygously deleted *DPC4* gene: Both types of cases showed complete absence of Dpc4 labeling in the nucleus and the cytoplasm. We did not have missense mutations available in our tumor panel, but if missense mutations were to occur in regions necessary for nuclear translocation of Dpc4, the mutations might result in intact cytoplasmic but absent nuclear labeling.

The immunohistochemical labeling of the invasive adenocarcinomas did not vary according to tumor type. That is, the two invasive adenocarcinomas arising within intraductal papillary mucinous neoplasms both had wild-type *DPC4* genes by genetic analysis, and both were labeled positively for the antibody. It is interesting that noninvasive components of both of these neoplasms, ranging from mild dysplasia to borderline changes to carcinoma *in situ*, all strongly expressed Dpc4. The two invasive adenocarcinomas of the distal common bile duct both had wild-type *DPC4* genes, and both were labeled positively for the antibody. The one ampullary adenocarcinoma had a homozygously deleted *DPC4* gene, and it showed no labeling for the Dpc4 protein. Finally, one pancreatic adenocarcinoma with a medullary phenotype had a homozygously deleted *DPC4* gene, and it showed no expression for the Dpc4 protein.¹⁵

Clinical and Pathological Data

Clinical, pathological, and genetic data on the 41 xenografted primary periampullary adenocarcinomas were collected and analyzed. There were no significant differences in any clinical, pathological, or genetic characteristic between patients whose tumors expressed (focally or diffusely) or did not express the Dpc4 protein. These characteristics included gender, race, age, family history of cancer, alcohol use, tobacco use, symptoms, comorbidities (see Materials and Methods), tumor differentiation, tumor size, tumor location, presence of nodal metastases at surgery, *K-ras* gene status, and microsatellite stability. In addition, although there was a

Table 1. Correlation of Final Genetic and Immunohistochemical Studies Including Genetic Reanalysis

	Immunohistochemical expression for Dpc4			
	Diffusely positive	Focally positive	Negative	Total
<i>DPC4</i> wild-type	16	1	1	18
<i>DPC4</i> HD	1	1*	18	20
<i>DPC4</i> mut/LOH	0	0	3	3
TOTAL	17	2	22	41

HD, homozygous deletion; mut/LOH, mutation coupled with loss of heterozygosity.

*This case was originally believed to be genetically wild-type, but on reanalysis was found to have an exon 0 mutation.

definite trend that patients whose tumors were labeled positively for Dpc4 had longer survival (mean/median survivals of 17.5/16.5 months) than those whose tumors were not labeled (mean/median survivals of 14.9/9.0 months), this difference did not reach statistical significance in this group of 41 patients ($P = 0.06$).

Discussion

Approximately 55% of pancreatic adenocarcinomas have inactivated both alleles of the *DPC4* gene. In 35% of the cancers, this inactivation occurs by homozygous deletion, and in 20% by mutation in one allele combined with loss of the other allele (loss of heterozygosity).⁵ Inactivation of the *DPC4* gene is relatively specific for pancreatic adenocarcinoma, although a minority of other cancer types, including mammary and colonic adenocarcinomas, has altered *DPC4* genes.^{1-3,6-10,16}

The *DPC4* gene produces a 64-kd protein that influences gene transcription and growth arrest.¹⁶⁻²² In fact, the Dpc4 protein has three distinguishable domains (for DNA binding, transcriptional activation, and nuclear translocation), and mutations in each these domains can lead to a loss of Dpc4 function.²³ Dpc4 mediates a transforming growth factor- β (TGF- β)-induced signaling pathway.²³⁻³³ Although it is generally held that Dpc4 is required for TGF- β -induced growth arrest, recent evidence shows that TGF- β sometimes may act in a Dpc4-independent manner.^{34,35}

The genetic analysis of an infiltrating cancer for *DPC4* alterations can be greatly limited by low neoplastic cellularity in a tumor. Therefore, we undertook this study to determine whether immunohistochemical analysis for the *DPC4* gene product could serve as a surrogate marker for *DPC4* gene alterations. Immunohistochemical analysis is technically less cumbersome and more widely available than are genetic assays, and it provides a way to correlate specific morphological and genetic changes in a large number of cases. If sensitive and specific, immunohistochemical analysis for *DPC4* gene expression could be used in both the clinical and investigative settings.

Immunohistochemistry for the *DPC4* gene product indeed proved to be a highly specific and sensitive marker for *DPC4* gene inactivation. Immunohistochemical labeling correctly identified the demonstrable *DPC4* gene status in 38 of 41 cancers (93%). The three discordant cases included one with a wild-type *DPC4* gene in the xenograft that did not express the Dpc4 protein in the primary carcinoma, one with a homozygously deleted *DPC4* gene in the xenograft that labeled diffusely with the antibody in the primary carcinoma, and one with a homozygously deleted *DPC4* gene in the xenograft that labeled focally with the antibody in the primary carcinoma. Another case with a wild-type *DPC4* gene in the xenograft focally expressed the Dpc4 protein in the primary carcinoma, and the immunohistochemical labeling and genetic analysis were considered concordant in this case.

There may be explanations for these genetic-immunohistochemical mismatches. The case with the wild-type *DPC4* gene that was negative for the Dpc4 protein may have involved inactivation of the *DPC4* gene through mechanisms other than homozygous deletion or mutation coupled with loss of heterozygosity. For example, mutation in or methylation of the *DPC4* promoter may turn off transcription of the gene.³⁶ Alternatively, it is possible that there was a mutation in a portion of the gene that had not been examined. For example, exon 0 of *DPC4* was discovered some years after the initial reports of *DPC4* inactivation in pancreatic adenocarcinomas (B Vogelstein and AB Sparks, unpublished data).

The positive labeling seen in the cancers with homozygously deleted *DPC4* genes is more difficult to explain. One explanation for positive labeling in these homozygously deleted cancers is cross-reactivity of the anti-Dpc4 antibody with another protein. A more intriguing explanation is that different areas of the neoplasms were sampled for genetic and immunohistochemical analyses, and it is possible that tumor heterogeneity, with *DPC4* loss being a late event in the portion of the tumor sampled for xenografting, may account for this discrepancy. Indeed, one of the two carcinomas that labeled only focally for Dpc4 had two distinct morphological patterns. The better differentiated glandular component expressed Dpc4, whereas the distinct, more poorly differentiated component did not (Figure 3D). It is possible that this more poorly differentiated pattern represents progression of the invasive cancer to a more aggressive phenotype, evidenced by both histology and genetics, and that it was this portion of the neoplasm that was sampled for xenografting. This progression would be similar to that seen in adenomas and carcinomas of the colon.³⁷⁻⁴⁰ It is also possible that two neighboring sites of invasive cancer could form a "collision tumor."

Finally, this study includes the first description of a deletion in exon 0 of the *DPC4* gene in a pancreatic cancer.

In summary, immunohistochemical labeling for the Dpc4 protein is an accurate and easy method to detect *DPC4* gene alterations with high sensitivity and specificity. In addition, immunohistochemical labeling provides information not obtainable through genetic analysis. For example, because it is performed *in situ*, immunohistochemistry may provide evidence for the heterogeneity of Dpc4 expression in a given carcinoma.

Because of its simplicity and availability, immunohistochemical labeling for Dpc4 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 (slightly more than half of which will not express Dpc4). In addition, the immunohistochemical assay for Dpc4 may lead to answers in the investigative arena. For instance, the immunohistochemical study of pancreatic duct lesions, the putative precursors for pancreatic adenocarcinoma, may help determine the stage at which *DPC4* inactivation contributes to neoplastic progression.

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