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Loss of Expression of Dpc4 in Pancreatic Intraepithelial Neoplasia: Evidence That *DPC4* Inactivation Occurs Late in Neoplastic Progression[1]

Robb E. Wilentz, Christine A. Iacobuzio-Donahue, Pedram Argani, Denis M. McCarthy, Jennifer L. Parsons, Charles J. Yeo, Scott E. Kern, and Ralph H. Hruban[2]

Departments of Pathology [R. E. W., C. A. I-D., P. A., D. M. M., J. L. P., S. E. K., R. H. H.], Oncology [C. J. Y., S. E. K., R. H. H.], and Surgery [C. J. Y.], The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287

ABSTRACT

Infiltrating adenocarcinomas of the pancreas are believed to arise from histologically identifiable intraductal precursors [pancreatic intraepithelial neoplasias (PanINs)] that undergo a series of architectural, cytological, and genetic changes. The role of *DPC4* tumor suppressor gene inactivation in this progression has not been defined. Immunohistochemistry for the Dpc4 protein in formalin-fixed, paraffin-embedded tissue is a sensitive and specific marker for *DPC4* gene status, providing a tool to examine *DPC4* status in these putative precursor lesions. A total of 188 PanINs were identified in 40 pancreata, 38 (95%) of which also contained an infiltrating adenocarcinoma. Sections containing these 188 duct lesions were labeled with a monoclonal antibody to Dpc4. All 82 flat (PanIN-1A), all 54 papillary (PanIN-1B), and all 23 atypical papillary (PanIN-2) intraductal lesions expressed Dpc4. In contrast, 9 of 29 (31%) severely atypical lesions (PanIN-3 lesions, carcinomas *in situ*) did not. The difference in Dpc4 expression between histologically low-grade (PanIN-1 and -2) and histologically high-grade (PanIN-3) duct lesions was statistically significant ($P < 0.0001$). In three cases, the pattern of Dpc4 expression in the PanIN-3 lesions did not match the pattern of expression in the associated infiltrating carcinomas, indicating that these high-grade lesions did not simply represent infiltrating carcinoma growing along benign ducts. Loss of Dpc4 expression occurs biologically late in the neoplastic progression that leads to the development of infiltrating pancreatic cancer, at the stage of histologically recognizable carcinoma.

INTRODUCTION

Recent evidence suggests that, in the pancreas, there is a neoplastic progression very similar to the adenoma-carcinoma sequence in the colon (1–4). That is, in some pancreatic ducts and ductules, a mucinous epithelium with cytological and architectural atypia replaces the normal cuboidal epithelium. These duct lesions are also known as PanINs,[3] and they are believed to progress from flat to papillary without atypia to papillary with atypia to carcinoma *in situ* (PanIN-1A to PanIN-1B to PanIN-2 to PanIN-3; Refs. 3–7). Some *in situ* lesions then eventually progress to infiltrating adenocarcinoma. Thus, even those clinically "early" infiltrating pancreatic cancers are, in fact, biologically late (4).

Infiltrating pancreatic cancers that are clinically early are also genetically late. Most infiltrating pancreatic cancers have accumulated numerous genetic alterations by the time they come to clinical presentation (8). For example, Rozenblum *et al.* (8) found nine separate key genetic alterations in a single infiltrating pancreatic cancer. Not surprisingly, PanINs also show many of these same genetic changes. For example, we and others (9–18) have identified K-*ras*, *HER-2/neu*, *p16*, *BRCA2*, and *p53* alterations in a variety of PanINs using both genetic and immunohistochemical analyses. By doing so, we have been able to establish the presumed relative timing of these genetic alterations in the pancreatic cancer progression model (see Table 1 for a literature review of gene alterations in PanINs).

Whereas alterations in these five genes have been studied in PanINs, inactivation of the *DPC4* tumor suppressor gene has not. *DPC4* is one of the major tumor suppressor genes targeted in infiltrating pancreatic adenocarcinoma. It is inactivated in over half of invasive pancreatic adenocarcinomas, and its inactivation is relatively specific for invasive pancreatic adenocarcinoma (19–21). That is, whereas many cancer types harbor alterations in the K-*ras*, *p16*, *BRCA2*, and *p53* genes, inactivation of *DPC4* occurs only infrequently in nonpancreatic cancers (19–26).

Therefore, we examined the expression of the Dpc4 protein in a spectrum of PanINs. We have recently shown that immunohistochemistry for Dpc4 is an extremely sensitive and specific marker for *DPC4* gene inactivation, independent of whether inactivation occurs by homozygous deletion (deletion of both alleles of the *DPC4* gene) or by mutation in one allele coupled with loss of the other allele (loss of heterozygosity; Ref. 17). Determining the patterns of Dpc4 expression in a large number of histologically defined PanINs will help to establish the role of *DPC4* inactivation in the development of pancreatic neoplasia.

MATERIALS AND METHODS

Specimen Selection. Pancreaticoduodenectomy specimens (Whipple resection specimens) from 40 patients were studied. Thirty-eight resections (95%) were performed for infiltrating adenocarcinoma, and two resections were performed for chronic pancreatitis. These resections fell into two groups: (*a*) 31 resections were originally selected for the study because the infiltrating adenocarcinoma in each of them had been previously analyzed genetically for *DPC4* gene mutations and analyzed immunohistochemically for Dpc4 expression (17, 19–21); and (*b*) 9 pancreata were added to the analyses because they contained well-defined, severely atypical intraductal neoplasias (PanIN-3 lesions, carcinomas *in situ*).

Identification of Duct Lesions. Multiple H&E-stained slides of pancreatic tissue from each of the cases were screened by light microscopy for PanINs. Criteria established at a National Cancer Institute-sponsored Pancreas Cancer Think Tank in September, 1999, in Park City, Utah were used to classify each lesion (3, 5–7, 27–29).[4] Unstained 5-μm sections containing the PanINs were then cut from paraffin blocks for immunohistochemical analysis.

Immunohistochemistry. Unstained sections were treated with a monoclonal antibody (clone B8; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) to Dpc4 under previously described conditions (17). Briefly, each slide was deparaffinzed by routine techniques, treated with sodium citrate buffer (diluted to 1× HIER buffer from 10× HIER buffer; Ventana-Bio Tek Solutions, Tucson, AZ), and steamed for 20 min at 80°C. After cooling for 5 min, the slides were labeled with the antibody using the Bio Tek-Mate 1000 automated stainer (Ventana-Bio Tek Solutions). The anti-Dpc4 antibody was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin. For negative controls, the primary antibody was replaced with normal saline.

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[2] To whom requests for reprints should be addressed, at Meyer 7-181, Department of Pathology, The Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287. Phone: (410) 955-9132; Fax: (410) 955-0115; E-mail: rhruban@jhmi.edu.

[3] The abbreviation used is: PanIN, pancreatic intraepithelial neoplasia.

[4] http://pathology.jhu.edu/pancreas_panin

Table 1 *Review of gene alterations in normal pancreatic ducts, (PanINs)*[a]*, and infiltrating pancreatic ductal adenocarcinomas*

Gene (reference no.)	Normal	PanIN-1A	PanIN-1B	PanIN-2	PanIN-3	Infiltrating
HER-2/neu (12)	5%	82%	86%	92%	100%	69%
K-*ras* (9, 13–15, 18, 32, 33)	0–15%[b]	35%	43%	[c]	86%	~90%
p16 (9, 10, 34, 35)	0%	24%	19%	55%	71%	~95%
p53 (11, 16, 36)	0%	0%	0%	[d]	21%	~75%
DPC4 (17, 19, this study)	0%	0%	0%	0%	31%	~55%
BRCA2 (31, 37)[e]	0%	0%	0%	0%	[F]	7%

[a] Histologic classification of PanINs is given in the text.
[b] Authors are not in agreement that normal ductal epithelium harbors K-*ras* mutations.
[c] Insufficient numbers of PanIN-2 were present in these studies to present an accurate result.
[d] One PanIN-2 identified by Hameed *et al.* (16) overexpressed p53, but the total number of PanINs-2 was not reported. Therefore, a percentage could not be calculated.
[e] *BRCA2* data in PanINs are restricted to patients with germ-line *BRCA2* inactivating mutations.
[f] Only one PanIN-3 was studied by Goggins *et al.*, (31) and it showed a *BRCA2* germ-line mutation coupled with loss of heterozygosity.

Immunohistochemical Interpretation. Two of the authors of this study (R. E. W. and R. H. H.) independently evaluated the immunohistochemical labeling of each PanIN, without knowledge of the gene status or expression phenotype of the associated infiltrating adenocarcinoma. The labeling of each lesion was scored as "positive," "weakly positive," or "negative" as described previously (17). Positive labeling was defined as strong and uniform expression of Dpc4 in the cytoplasm of the majority of cells, with focal expression of Dpc4 in nuclei. Weakly positive PanINs showed weak expression of Dpc4 in the cytoplasm of the majority of cells, with or without 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. Positive and weakly positive cases were grouped together as positive for subsequent analysis.

The interpretation of immunohistochemical labeling of the lesions was highly robust, with agreement between the observers in 185 of the 188 (98%) PanINs. Two of the three discordant cases were called positive by one author and weakly positive by the other. The third PanIN was interpreted as weakly positive by one author and as negative by the other. Re-examination of these cases by both authors together resulted in agreement that the former PanINs were weakly positive and that the latter was negative.

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

Statistical Analysis. The presence of Dpc4 expression in PanINs was analyzed with two-tailed Fisher's exact tests. Tests were performed using Statistica for Windows (StatSoft, Tulsa, OK).

RESULTS

Associated Infiltrating Adenocarcinomas. Thirty-one of the 40 resections included in this study contained infiltrating adenocarcinomas previously analyzed genetically for *DPC4* mutations and immunohistochemically for Dpc4 expression (17, 19–21). Of these 31 infiltrating adenocarcinomas, 25 were conventional ductal adenocarcinomas of the pancreas, 1 was a poorly differentiated adenocarcinoma of the pancreas with clear cell features, 4 were adenocarcinomas of the distal common bile duct, and 1 was an adenocarcinoma of the papilla (ampulla) of Vater.

This group of 31 cancers included 20 infiltrating cancers with inactivated *DPC4* genes and 11 cancers with at least one wild-type *DPC4* allele. Of the 20 cancers with inactivated genes, 19 had homozygously deleted genes, and 1 had a mutant allele coupled with loss of the other allele (loss of heterozygosity). As reported previously (17), all 11 of the infiltrating cancers with wild-type alleles showed Dpc4 expression by immunohistochemistry. Eighteen of the 19 (95%) infiltrating cancers with homozygously deleted genes did not express Dpc4, and the one cancer with a mutant allele coupled with loss of heterozygosity also did not express Dpc4. Therefore, our previous study showed that immunohistochemistry detects inactivation of the *DPC4* gene, regardless of the mechanism of inactivation (17).

Nine pancreata were added to the study because they harbored well-defined PanIN-3 lesions; seven (78%) of these also had separate areas of infiltrating adenocarcinoma. Six (86%) of the infiltrating cancers were conventional ductal adenocarcinomas of the pancreas, and one was an adenocarcinoma of the distal common bile duct. Four of these seven (57%) infiltrating cancers, including the one originating in the common bile duct, expressed the Dpc4 protein. The two pancreata with only chronic pancreatitis had been entirely submitted for histological examination to rule out foci of invasive cancer.

Thus, 38 of the 40 (95%) pancreata included in this study harbored an infiltrating adenocarcinoma, and 22 of these 38 (58%) infiltrating cancers showed loss of Dpc4 expression.

PanINs. A total of 188 PanINs were identified in the 40 pancreata included in this study. Overall, 179 (95%) of these PanINs expressed Dpc4. All 82 PanIN-1As, all 54 PanIN-1Bs (Figs. 1 and 2), and all 23

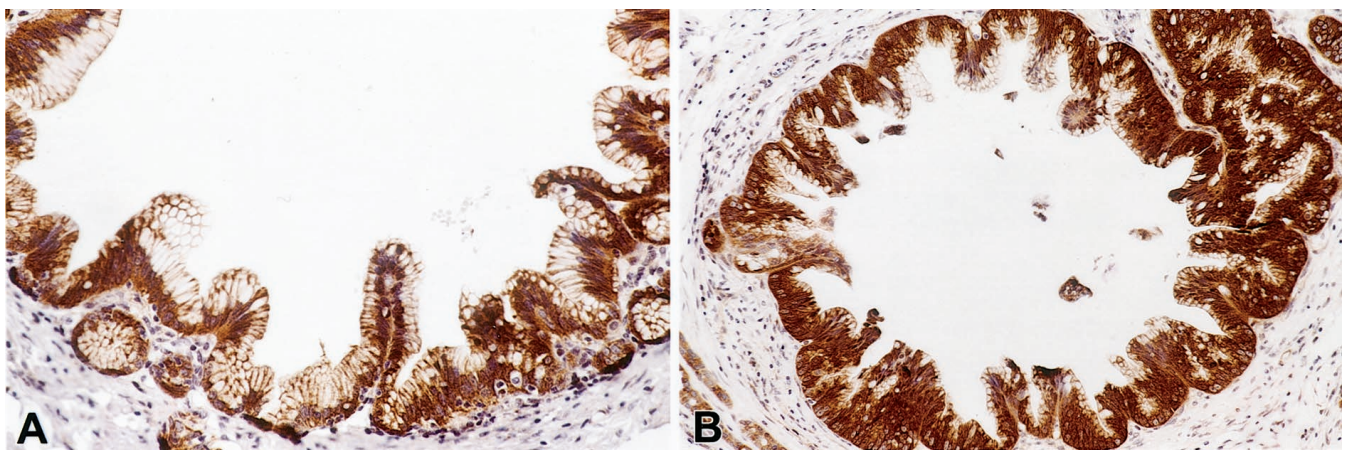

Fig. 1. *A*, a PanIN-1B expresses the Dpc4 protein in both the cytoplasmic and nuclear compartments. All PanIN-1Bs in this study expressed the Dpc4 protein. *B*, a PanIN-3 has strong and uniform expression of the Dpc4 protein.

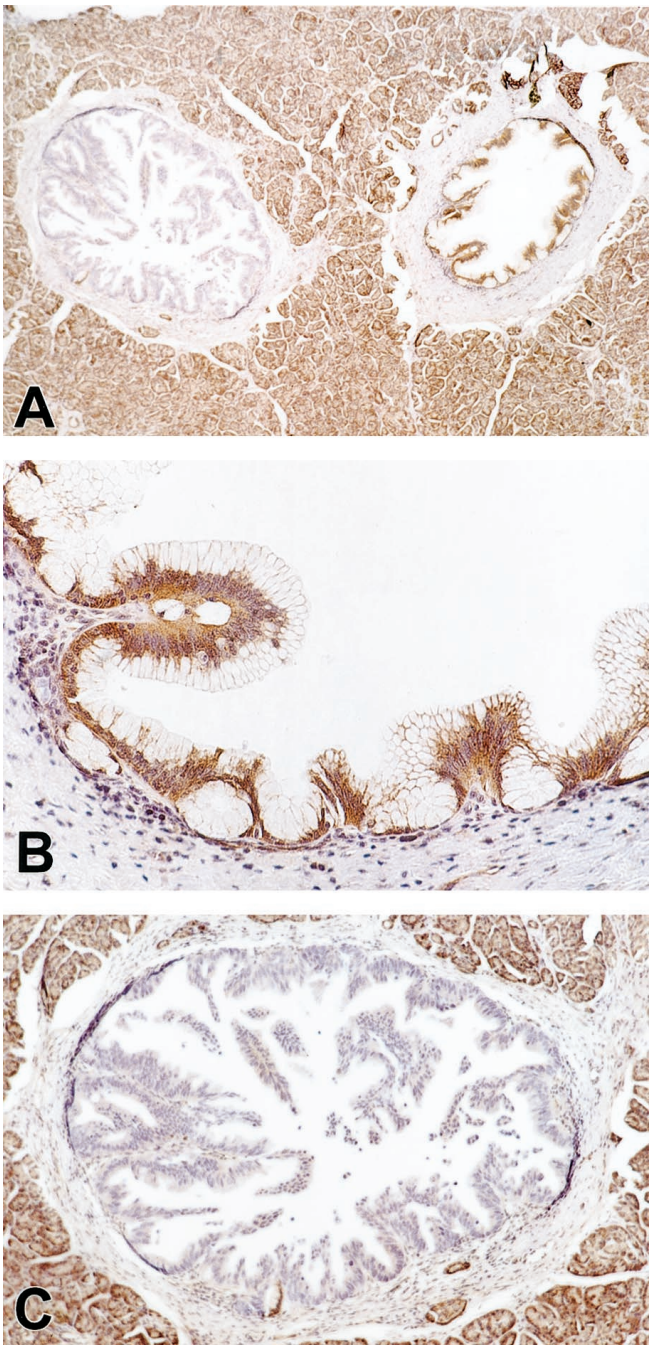

Fig. 2. *A*, a PanIN-1B (*right*) and a PanIN-3 (*left*) from the same pancreas. The PanIN-1B expresses Dpc4, but the PanIN-3 does not. *B*, higher power view of the PanIN-1B. *C*, higher power view of the PanIN-3.

PanIN-2s expressed Dpc4 (Table 2). In contrast, Dpc4 expression was found in 20 of the 29 (69%) PanIN-3s (Figs. 1 and 2). The difference in Dpc4 expression between "early" (PanIN-1A, PanIN-1B, and PanIN-2) and "advanced" (PanIN-3) duct lesions was statistically significant ($P < 0.0001$).

There was no progressive weakening in intensity of Dpc4 expression from early PanINs to advanced PanINs to invasive carcinomas. Whereas both strongly and weakly positive cases were indeed grouped together for analysis, it is important to note that the overwhelming majority of PanINs that express Dpc4 do so strongly. For example, 52 of the 52 (100%) PanIN-1As, 50 of the 54 (93%) PanIN-1Bs, and 22 of the 23 (96%) PanIN-2s included in this study labeled strongly for Dpc4. In addition, of the 20 PanIN-3s that expressed Dpc4, 18 (90%) did so strongly. Finally, a previous study on Dpc4 expression in infiltrating carcinomas revealed that 17 of 19 (90%) infiltrating carcinomas expressing Dpc4 labeled strongly with our technique (17).

Evaluation of Dpc4 Expression in PanIN-3s (Carcinomas *in Situ*). Twenty-nine PanIN-3s were identified in 12 pancreata (see Table 3). Twenty (69%) of these lesions expressed Dpc4, and nine (31%) did not. The 20 PanIN-3s that expressed Dpc4 were from eight different patients. The eight resections included the following: (*a*) three with associated infiltrating pancreatic ductal adenocarcinomas that expressed Dpc4; (*b*) two with associated infiltrating pancreatic ductal adenocarcinomas that did not express Dpc4; (*c*) one with an associated infiltrating pancreatic ductal adenocarcinoma that focally expressed Dpc4; (*d*) one with an associated infiltrating distal common bile duct adenocarcinoma that expressed Dpc4; and (*e*) one without an infiltrating carcinoma.

Nine of the 29 (31%) PanIN-3s did not express Dpc4, and these originated in four patients. Within these four pancreata, 11 PanIN-1As, 3 PanIN-1Bs, and 2 PanIN-2s, all expressing Dpc4, were also identified. These four resections included the following: (*a*) two with associated infiltrating pancreatic ductal adenocarcinomas that did not express Dpc4; (*b*) one with an associated infiltrating distal common bile duct adenocarcinoma that expressed Dpc4; and (*c*) one without an infiltrating carcinoma. Table 3 summarizes the results from all of the cases containing at least one PanIN-3.

Thus, in two cases with PanIN-3, there was no associated infiltrating cancer. In 7 of the 10 (70%) remaining cases, the pattern of Dpc4 expression in the PanIN-3s matched the pattern of Dpc4 expression in the associated infiltrating carcinomas. In contrast, in three cases, the pattern of labeling in the PanIN-3s did not match that in the associated infiltrating cancers. These findings demonstrate that these PanIN-3s were not simply an artifact of an infiltrating carcinoma extending along preexisting benign ducts.

DISCUSSION

Pancreatic adenocarcinoma is a genetic disease. For example, approximately 55% of pancreatic adenocarcinomas show inactivation of the *DPC4* gene (17, 19–21). Homozygous deletion (deletion of both alleles) inactivates *DPC4* in 35% of pancreatic adenocarcinomas, and intragenic mutation in one allele coupled with loss of the second allele (loss of heterozygosity) inactivates it in another 20% of pancreatic adenocarcinomas (19–21).

Genetic changes are also seen in PanINs, the putative precursors of infiltrating duct adenocarcinoma of the pancreas (9–11). These precursors can show a spectrum of architectural and cellular changes. They can be flat (PanIN-1A), papillary (PanIN-1B), papillary with moderate atypia (PanIN-2), or severely atypical (PanIN-3, carcinoma *in situ*; Refs. 3–5, 7, and 27–29; see Fig. 3). Alterations in the sequences or expression levels of the K-*ras*, *HER-2/neu*, *p16*, *BRCA2*, and *p53* genes have already been detected in PanINs, and the prevalence of these mutations increases with increasing grade of the PanIN (Refs. 9–18; see Table 1). If we assume that the higher-grade PanINs represent more advanced lesions than the lower-grade PanINs, then

Table 2 *Dpc4 expression in subtypes of PanINs*[a]

Expression	PanIN-1A	PanIN-1B	PanIN-2	PanIN-3	Total
Positive	82	54	23	20	179
Negative	0	0	0	9	9
Total	82	54	23	29	188

[a] Histological classification and immunohistochemical interpretations are given in the text. $P < 0.0001$ (PanIN-1 and 2 *versus* PanIN-3, Fisher's exact test).

Table 3 *Catalogue of cases with PanIN-3*[a]

Case	No. of PanINs-3 with Dpc4 expression	No. of PanINs-3 without Dpc4 expression	Associated invasive carcinoma	Dpc4 expression in associated invasive carcinoma	Other PanINs identified[b]
1	4	0	Pancreatic[c]	Present	None
2	1	0	Pancreatic	Present	None
3	2	0	Pancreatic	Present	1 PanIN-1B and 1 PanIN-2
4	3	0	Pancreatic	Absent	None
5	1	0	Pancreatic	Absent	None
6	1	0	Pancreatic	Focally present	2 PanIN-1As
7	2	0	Bile duct[d]	Present	2 PanIN-2s
8	6	0	None	NA[e]	None
9	0	1	Pancreatic	Absent	None
10	0	1	Pancreatic	Absent	8 PanIN-1As and 2 PanIN-2s
11	0	5	Bile duct	Present	3 PanIN-1As and 2 PanIN-1Bs
12	0	2	None	NA	1 PanIN-1B

[a] Histological classification of PanINs is given in the text.
[b] All of the PanIN-1As,-1Bs, and -2s expressed Dpc4.
[c] "Pancreatic" refers to ductal adenocarcinomas originating within the pancreas.
[d] "Bile duct" refers to adenocarcinomas arising within the distal common bile duct.
[e] NA, not applicable.

these data help establish the relative timing of genetic alterations in the progression model of pancreatic adenocarcinoma.

Whereas the prevalence of alterations in these five genes has been studied in various grades of PanIN, the prevalence of *DPC4* alterations has not. We therefore examined the expression of the Dpc4 protein in a spectrum of PanINs. We chose this indirect immunohistochemical method because we have previously shown that immunohistochemistry for Dpc4 is an extremely sensitive and specific marker for *DPC4* gene status (17).

A total of 188 PanINs were identified in the 40 pancreata included in this study. All (100%) of the PanIN-1As, PanIN-1Bs, and PanIN-2s expressed Dpc4. In contrast, approximately one-third of the PanIN-3s (severely atypical duct lesions, carcinomas *in situ*) lost Dpc4 expression. The difference in Dpc4 expression between the former and the latter was statistically significant ($P < 0.0001$).

These data lead to several striking conclusions. First, and most obviously, inactivation of the *DPC4* gene occurs late in the development of pancreatic adenocarcinoma, at the stage of *in situ* or even invasive carcinoma. That is, loss of Dpc4 is synonymous with the onset of carcinomatous change. This fact has important implications for tumor biology because it shows that dramatic biological alterations may be strongly associated with the loss of a particular gene. Our findings also should stimulate the creation of experimental models that investigate the molecular basis of the transition between "benign" and "malignant," similar to *DPC4-APC* compound mutant mice that have already been developed (30).

Second, because inactivation of *DPC4* occurs late in the progression of pancreatic adenocarcinoma, immunohistochemistry for Dpc4 may be useful as a clinical marker for pancreatic carcinoma. The loss of Dpc4 expression appears to be very specific for highly advanced neoplasia/carcinoma. Conversely, however, because only 55% of invasive pancreatic adenocarcinomas show inactivation of the *DPC4* gene, the presence of intact Dpc4 cannot be used to indicate that a lesion is benign.

Third, these data help establish that the lesions we have designated as PanIN-3s do not simply represent a "cancerization of benign ducts." In three of the cases we examined, the pattern of Dpc4 expression in the PanIN-3s (carcinomas *in situ*) did not match the pattern of Dpc4 expression in the associated infiltrating adenocarcinomas, and in two additional cases, an infiltrating carcinoma was not present. Therefore, these PanIN-3s (carcinomas *in situ*) could not simply be a manifestation of infiltrating cancer growing along preexisting ducts. While cancerization of benign ducts does occur within the pancreas, our data show that malignant-appearing cells within a duct do not always reflect such a process. Instead, PanIN-3s may represent lesions independent from an associated infiltrating carcinoma. In addition, the case with a Dpc4-positive biliary carcinoma and a Dpc4-negative PanIN-3 suggests that although pancreatic and biliary neoplasia can occur together, they may be genetically distinct.

Finally, these data and previous data allow us to begin to construct a genetic model of pancreatic cancer progression (Table 1; Fig. 3). K-*ras* mutations and HER-2/neu overexpression are the earliest changes in the progression model (9, 12–15, 18). Alterations in *p16* occur at different histological stages but are found primarily in PanIN-2s and PanIN-3s (9, 10). *DPC4*, *BRCA2*, and *p53* appear to be inactivated very late in the progression model (11, 16, 31).

Clearly, this progression model of pancreatic neoplasia is still under construction. More genes may be identified, and as greater numbers of cases are examined, the timing of the known alterations may be more accurately determined. Nevertheless, it is clear that *DPC4* genetic

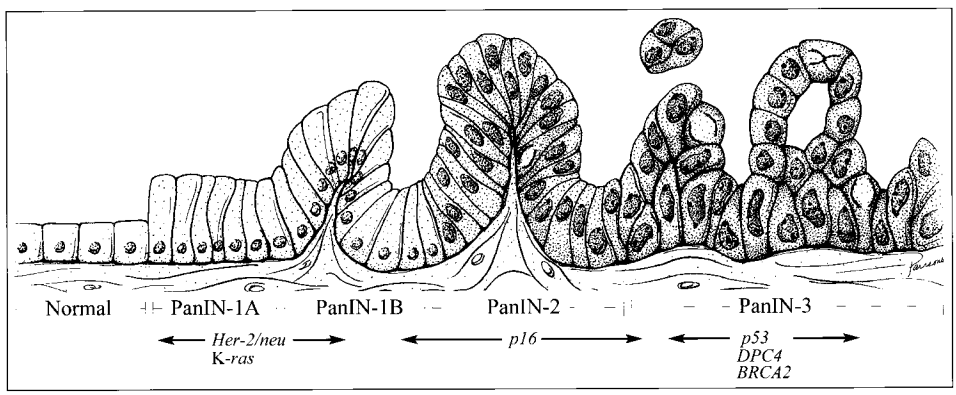

Fig. 3. Progression model for duct adenocarcinoma of the pancreas. It is hypothesized that a duct lesion can progress from a histologically normal duct to flat duct lesion (*PanIN-1A*) to papillary duct lesion (*PanIN-1B*) to atypical papillary duct lesion (*PanIN-2*) to severely atypical duct lesion/carcinoma *in situ* (*PanIN-3*). The approximate timing of alterations in the K-*ras*, *HER-2/neu*, *p16*, *p53*, *BRCA2*, and *DPC4* genes is indicated on the model (see also Table 1; artwork by Jennifer L. Parsons).

alterations are important in the progression to carcinoma and that immunohistochemistry provides an indirect but effective and convenient means to determine *DPC4* expression in a large number of cases.

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Robb E. Wilentz, Christine A. Iacobuzio-Donahue, Pedram Argani, et al.

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