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Advances in Brief

p16 and K-*ras* Gene Mutations in the Intraductal Precursors of Human Pancreatic Adenocarcinoma[1]

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Abstract

Pancreatic adenocarcinoma is thought to arise from a noninvasive neoplastic precursor, the pancreatic intraductal lesion (PIL). Mutations of the K-*ras* gene are known to occur in PILs, but their high prevalence among PILs within the general population probably limit the use of K-*ras* as a marker of eventual clinical risk. In search of genetic constellations that might indicate the progression of some PILs toward an invasive phenotype, mutations at both the K-*ras* and *p16* genes were sought within PILs of 10 pancreata resected for adenocarcinoma. K-*ras* mutations were present in most PILs and in nearly all PILs having nuclear atypia. In half of the patients, two or more unique K-*ras* mutations were identified among distinct PILs, which is evidence for the separate clonal evolution of multiple pancreatic neoplasms within individual patients. *p16* alterations (one homozygous deletion and three point mutations) were found in 4 of the 10 carcinomas; these four pancreata harbored *p16* alterations in three of nine PILs, of which one was a "histologically early" lesion. Two patients had *p16* alterations in PILs matching those of the associated carcinomas. *p16* mutations were not found in PILs of pancreata having wild-type *p16* in the carcinoma, nor were they found in ducts having normal histology. It is suggested that alterations of the *p16* gene affect a subset of PILs that contain mutations of the K-*ras* gene and that these mutations might identify high-risk precursors of the invasive malignancy.

Introduction

The nature of the precursors of various epithelial malignancies has been addressed by a combination of histological, epidemiological, and molecular approaches (1–3). In general, the precursors of a carcinoma can be identified microscopically, with their stages of histological progression correlating with the risk of invasion. Molecular genetic studies have shown that the precursors have some of the same mutations as the invasive carcinomas. For example, in the adenoma-carcinoma sequence of colorectal tumorigenesis, it has been demonstrated that the (benign) adenomas frequently share the same genetic lesions as the (malignant) carcinomas, such as mutations of the *APC*, K-*ras*, and *p53* genes (4). A greater number of genetic mutations can be found in larger, histologically advanced adenomas as compared to the small, histologically better-differentiated adenomas, supporting a concept of molecular tumor progression in which invasive carcinoma occurs as the culmination of a microevolutionary accumulation of mutations in clonal populations of epithelial cells.

There have been several histological studies of epithelial changes within the pancreatic ducts associated with infiltrating adenocarcinoma of the pancreas. These include alterations of cell shape (usually from low cuboidal to tall columnar morphology), differentiation (often from low mucin content to hypermucinous cells), and growth pattern (most commonly from a "flat" single cell layer to a papillary or pseudopapillary pattern having tuft-like projections of cells into the duct lumen). Several workers have categorized these lesions as "hyperplasia" (nonneoplastic), "atypical hyperplasia," or "carcinoma *in situ*" (neoplastic; Refs. 5–8) on the basis of the severity of histological changes. Others have labeled every deviation from the normal cuboidal epithelium as "dysplasia" (by convention, a term used for neoplastic epithelium; Ref. 9) and have also graded these lesions on the basis of the severity of histological changes (10, 11). It has been difficult to reconcile these various definitions or, indeed, to find agreement on what changes constitute the earliest neoplastic precursors of adenocarcinoma of the pancreas. Thus, the ability of histopathology to reproducibly separate these lesions into nonneoplastic and neoplastic categories has been called into question (12–14). For research purposes, we use the noncommittal term PIL[4] to describe any deviation from normal histology of the pancreatic duct epithelium and use the descriptive term "nuclear atypia" for PILs displaying distinctive and defined alterations in nuclear morphology.

The genetic alterations present in infiltrating pancreatic adenocarcinoma have been well characterized, and these include frequent mutation of the K-*ras* and *p16* genes and, somewhat less frequently, the *p53* and *DPC4* genes (15, 16). K-*ras* mutations have also been detected in PILs, and this genetic lesion appears to be a very early event in pancreatic tumorigenesis (14, 17–20). Other genetic changes in PILs have not been reported. Here, we perform a joint analysis of the K-*ras* and *p16* genes in PILs microdissected from human pancreata that had given rise to invasive adenocarcinoma.

Materials and Methods

Histological slides of pancreatic resection specimens from the surgical pathology files of The Johns Hopkins Hospital were reviewed, and 10 cases were selected on the basis of the presence of infiltrating pancreatic adenocarcinoma of the pancreas and the availability of archival material adequate for the study of associated PILs. A PIL was defined as a focus of tall columnar epithelial cells confined to a pancreatic duct. PILs were classified as either flat or papillary on the basis of growth pattern, and nuclear morphology was graded as normal, mild atypia, or severe atypia. A papillary growth pattern was defined as a deviation from the normal round or oval outline of pancreatic ducts by lumenal protrusions of groups of epithelial cells, with or without fibrovascular cores. The histological criteria for nuclear atypia were as follows: no atypia, small, round, basally located nuclei, similar or identical to those found in ducts judged to have normal epithelium; mild atypia, crowded, predominantly basally located enlarged nuclei, with either a vesicular, clumped chromatin pattern or a dense hyperchromatic chromatin pattern, round or oval in shape, with or without nucleoli; and severe atypia, enlarged nuclei with irregular contours, displaying cell-to-cell variability, hyperchromasia, loss of polarity, with or without nucleoli. The histological features were graded by two pathologists (C. A. M. and R. H. H.) prior to the molecular analysis.

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[4] The abbreviation used is: PIL, pancreatic intraductal lesion.

Adjacent 7-μm sections from formalin-fixed, paraffin-embedded tissue blocks were stained with H&E. PILs, infiltrating carcinoma, and adjacent normal tissue were dissected under direct visualization using an inverted microscope and a glass needle attached to a micromanipulator (21). An example of a dissected tissue focus is shown in Fig. 1. DNA was extracted from this tissue and subjected to PCR amplification of the region of the K-*ras* gene containing codons 12 and 13 and exons 1 and 2 of the *p16* gene, as described (14, 22). PCR products were subsequently analyzed by DNA cycle sequencing as described (14, 22). To control for the quality of template DNA in the samples, a 500-bp sequence from an area known to be infrequently deleted in pancreatic carcinoma (DPC2'; Ref. 23) was used as a control target of amplification in duplex PCR assays of the *p16* gene.

Results

K-*ras* Mutational Analysis. The region encompassing codons 12 and 13 of the K-*ras* gene was successfully amplified from all microdissected tissue foci. K-*ras* mutations were found in all invasive adenocarcinomas (10 of 10) and in at least one PIL from each patient (Table 1 and Fig. 2). In total, 24 histologically distinct and anatomically discrete PILs were examined in this manner, and a K-*ras* mutation was identified in 75% (18 of 24). All mutations were confirmed by repeat PCR and DNA cycle sequencing. K-*ras* mutations were highly associated with atypical nuclear features ($P < 0.0001$, Fisher exact test) and were always present in lesions having an intraductal papillary growth pattern (12 of 12). No K-*ras* mutations were detected in histologically normal pancreatic epithelium (0 of 10). K-*ras* mutations were detected in 29% of PILs (2 of 7) lacking a papillary growth pattern or atypia (designated "F" in Table 1). In only 6% of PILs with atypia (1 of 18) was a mutant sequence absent at codons 12 and 13.

In 5 of 10 pancreata examined, different K-*ras* mutations were

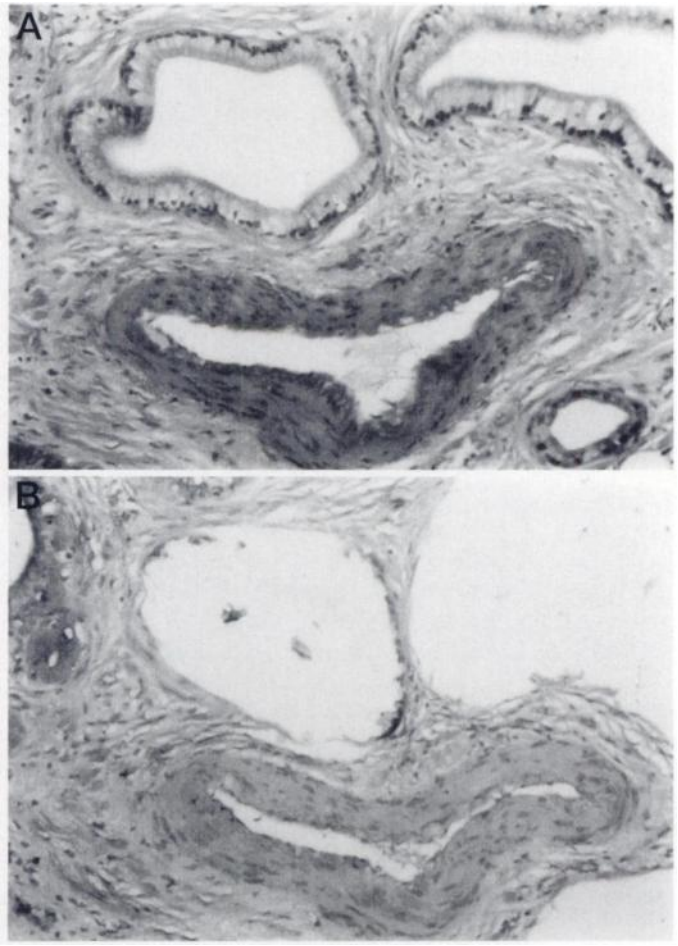

Fig. 1. Example of microdissection. Pancreatic ducts containing a nonpapillary (flat) PIL, with nuclei judged to have no atypical features (Table 1, case 5, F) present in the histological section (*A*) adjacent to the section used in microdissection (*B*). H&E, ×200.

Table 1 *Mutation status of tissue foci microdissected from histologic sections of human pancreata*

Specimen designation[a]	Status[b] K-*ras*	Status[b] *p16*
Case 1		
NNP[c]	WT	WT
NP	WT	WT
F	WT	WT
P-SA	Codon 12 GAT	Codon 100 asp to tyr
C	Codon 12 GAT	Codon 100 asp to tyr
Case 2		
NNP	WT	WT
NP	WT	WT
F-MA	Codon 12 GAT	WT
P-MA	Codon 12 GAT	WT
C	Codon 12 GTT	Codon 112 glu to stop
Case 3		
NNP	WT	WT
NP	WT	WT
F-MA	Codon 12 GTT	WT
P-MA	Codon 12 GTT	WT
C	Codon 12 GTT	WT
Case 4		
NNP	WT	WT
NP	WT	WT
F	WT	WT
P-SA	Codon 12 GTT	NA
C	Codon 12 GTT	WT
Case 5		
NNP	WT	WT
NP	WT	WT
F	Codon 12 GTT	Codon 100 asp to his
P-SA	Codon 12 GTT	Codon 84 asp to asn
C	Codon 12 GTT	Codon 100 asp to his
Case 6		
NNP	WT	WT
NP	WT	WT
F	WT	WT
F-MA	Codon 12 AGT	WT
P-MA	Codon 12 AGT & GTT	WT
C	Codon 12 GTT	WT
Case 7		
NNP	WT	WT
NP	WT	NA
P-SA	Codon 12 GTT	WT
C	Codon 12 GTT	WT
Case 8		
NNP	WT	WT
NP	WT	NA
F	WT	WT
P-MA	Codon 12 GTT	WT
P-SA	Codon 12 GAT	WT
C	Codon 12 GAT	NA
Case 9		
NNP	WT	WT
NP	WT	WT
F	WT	WT
F-MA	Codon 12 AGT	WT
P-MA	Codon 12 CGT	WT
P-SA	Codon 12 CGT	NA
C	Codon 12 CGT	Homozygous deletion
Case 10		
NNP	WT	NA
NP	WT	WT
F	Codon 12 CGT	WT
F-MA	WT	NA
P-MA	Codon 12 CGT	WT
C	Codon 12 GAT	WT

[a] Each case number (1–10) refers to a single resection specimen.

[b] Mutations are shown with the number of the codon involved and the sequence of the mutated codon (K-*ras*) or the presumptive change in amino acid coding (*p16*).

[c] NNP, normal tissue (not pancreatic duct epithelium); NP, normal pancreatic duct epithelium; F, nonpapillary (flat) PIL without atypia; F-MA, flat PIL with mild atypia; P-MA, papillary PIL with mild atypia; P-SA, papillary PIL with severe atypia; C, carcinoma; WT, wild-type sequence; NA, *p16* data not available, due to DNA degradation in samples, as confirmed by a control reaction.

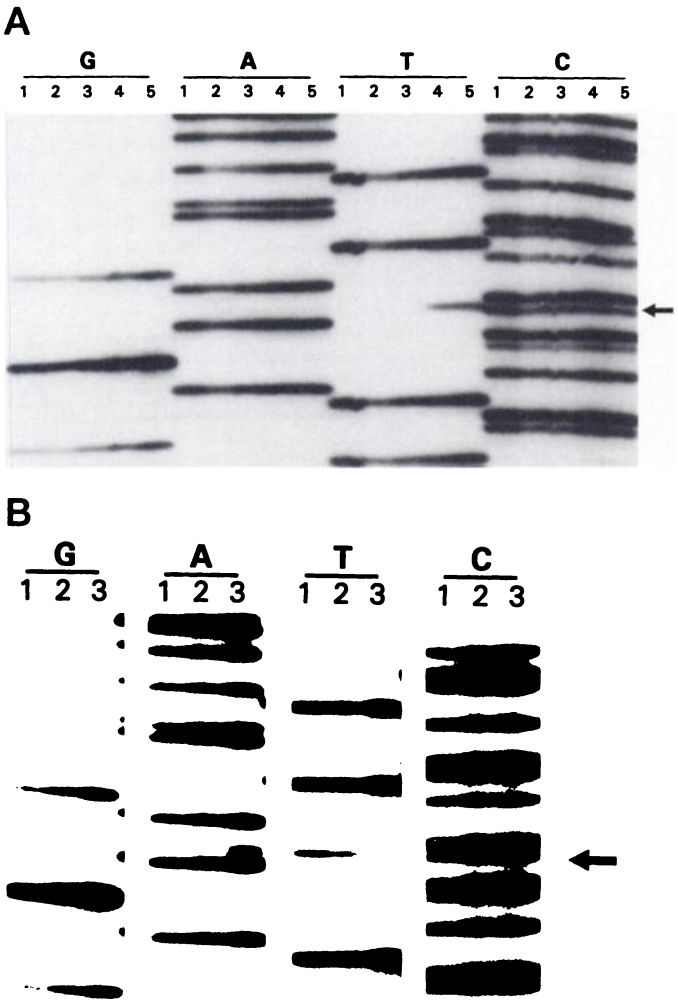

Fig. 2. Examples of cycle sequencing of K-*ras* PCR products. The nucleotide designation (G, A, T, C) refers to the sequenced antisense strand. *A*, *Lanes 1*, case 1, normal duodenal epithelium; *Lanes 2*, normal pancreatic duct epithelium; *Lanes 3*, flat PIL without atypia; *Lanes 4*, papillary PIL with severe atypia; *Lanes 5*, adenocarcinoma. *Arrow*, mutation of codon 12 [GGT to GAT (sense), Gly to Asp], present in the papillary PIL and in the carcinoma. *B*, *Lanes 1*, case 2, flat PIL with mild atypia; *Lanes 2*, papillary PIL with mild atypia; *Lanes 3*, adenocarcinoma. *Arrow*, same mutation of PILs in codon 12 [GGT to GAT (sense), Gly to Asp], which differs from the codon 12 mutation present in the carcinoma [GGT to GTT (sense), Gly to Val].

identified either among the anatomically distinct PILs and/or between the PILs and the infiltrating adenocarcinoma. In five cases (Table 1, cases 2, 6, 8, 9, and 10), the K-*ras* mutation found in the invasive adenocarcinoma was different from the mutation present in at least one of the PILs (Fig. 2). In three cases (Table 1, cases 6, 8, and 9), anatomically discrete PILs were found to have different K-*ras* mutations. In one instance, microdissection of a single small duct with severe atypia was found to harbor two K-*ras* mutations (Table 1, case 6, P-MA).

***p16* Mutational Analysis.** Exons 1 and 2 of the *p16* gene were amplified and sequenced in 45 of 54 (83%) microdissected foci. Of the nine samples in which the *p16* sequences did not amplify, eight also did not amplify the 500-bp control fragment in duplex PCR. These cases were considered as failed reactions due to degradation of target DNA sequences. The ninth case was a microdissected adenocarcinoma (Table 1, case 9), from which the 500-bp control sequence was amplified in duplex PCR, but the 246-bp PCR product of the *p16* exon 1 sequence failed to amplify in two separate simple and duplex reactions (Fig. 3). These findings are consistent with homozygous deletion of the *p16* gene in this adenocarcinoma.

In addition to the adenocarcinoma with homozygous deletion of the *p16* gene, three additional adenocarcinomas were found to have intragenic somatic mutations of the *p16* gene (Table 1, cases 1, 2, and 5). Two of these pancreata (cases 1 and 5) were found to have PILs that harbored the same *p16* mutation as the infiltrating carcinoma. One case (case 5) was found to have two histologically distinct PILs, separated by less than 1 cm, which contained different *p16* mutations (codon 100, GAT to CAT, and codon 84, GAC to AAC; Table 2 and Fig. 4). All intragenic *p16* mutations in PILs had evidence of loss of heterozygosity, as shown by a relative decrease in the signal of the wild-type allele in sequence analyses. The PIL that contained the same *p16* mutation as the infiltrating carcinoma did not exhibit nuclear atypia (Fig. 1).

Discussion

PILs are common in pancreata with either benign diseases or malignant tumors (24). Indeed, some evidence suggests that the majority of the adult human population with nondiseased pancreata harbor PILs (12, 19). K-*ras* mutations are present in the vast majority of infiltrating adenocarcinomas of the pancreas (25) and form a "signature mutation" of this disease. Hence, the presence of K-*ras* mutations has been sought in the precursor lesions to pancreatic adenocarcinoma and has been found in PILs obtained from both diseased and nondiseased pancreata (14, 17–20). These findings support the concept that some PILs are neoplastic. However, PILs with histological changes not previously regarded as neoplastic in nature have also been found to have K-*ras* mutations, and PILs containing K-*ras* mutations are much more common than are infiltrating adenocarcinomas of the pancreas. These findings have lead some to question the significance of K-*ras* mutation as a predictive test for the development of pancreatic adenocarcinoma (14, 19). Previously, there has been no molecular genetic analyses of PILs, other than those of the K-*ras* gene, reported.

Our findings of K-*ras* mutations in the majority of PILs and the demonstration of *p16* alterations in a subset of PILs extend the tumor progression model of pancreatic adenocarcinoma. (*a*) Our data show that more than one genetic lesion is present in some PILs and that these genetic lesions can be identical to those of the adjacent carcinomas. This unequivocally establishes PILs as precursor lesions to pancreatic adenocarcinoma. (*b*) We show that at least 50% of pancreata with adenocarcinoma contain distinct clonal populations of epithelial cells with differing K-*ras* mutations. This corroborates previous observations that K-*ras* mutation occurs frequently within pancreatic ducts and lends weight to the supposition that PILs with K-*ras* mutations do not inevitably culminate in invasive carcinoma. (*c*) Our data suggest that mutations of the K-*ras* gene might occur earlier than those of the *p16* gene in pancreatic tumor progression for the following reasons: the activating mutations of the K-*ras* gene occur more often than do the inactivating mutations of the *p16* gene in PILs; in no instance did we identify a PIL that contained a *p16* mutation that did not also contain a K-*ras* mutation; in pancreata that harbored *p16* mutations, more PILs were found that had K-*ras* mutations than had *p16* lesions; and in none of the histologically normal pancreatic duct epithelium sampled was a *p16* mutation identified. The detection of *p16* mutations in PILs, together with evidence of loss of the wild-type *p16* allele, suggests that there was clonal selective pressure for these mutations within the intraductal stages of pancreatic tumorigenesis.

Because of the low discriminating ability of K-*ras* mutations for malignant potential, it is important to ascertain other genetic markers that may better identify the lesions that harbor the greatest clinical risk. Interestingly, in at least in one case (Fig. 1, case 5), the presence

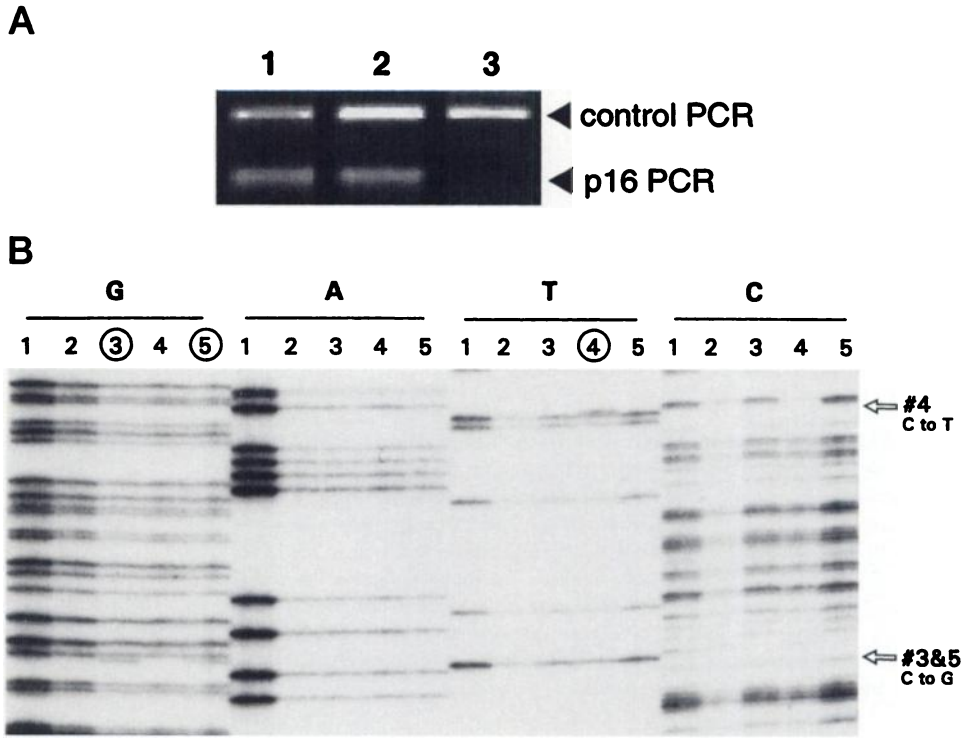

Fig. 3. Genetic alterations of the *p16* gene in PILs and pancreatic adenocarcinoma. *A*, samples of adenocarcinoma from case 9 repeatedly failed to amplify 246- and 469-bp PCR products from *p16* sequences. As a control for DNA template quality, duplex PCR assays were carried out using primers that amplify a 500-bp control PCR product, as well as exon 1 of the *p16* gene (246 bp). Genomic DNA from an unrelated person (*Lane 1*), and samples of normal pancreatic duct epithelium from case 9 (*Lane 2*) amplify both the control and *p16* sequences, but the adenocarcinoma sample (*Lane 3*) failed to coamplify the *p16* sequence, indicating a deletion of the target DNA from the tumor genome. *B*, cycle sequencing of *p16* products from case 5 (antisense strand, exon 2). *Lanes 1*, normal duodenal epithelium; *Lanes 2*, normal pancreatic duct epithelium; *Lanes 3*, flat PIL without atypia *Lanes 4*, papillary PIL with severe atypia; *Lanes 5*, adenocarcinoma. Bands corresponding to mutant alleles can be seen in the G lanes from samples of the flat PIL and adenocarcinoma (G *Lanes 3* and *5*, *circled*; *lower arrow*, position on gel) and in the T lane of the papillary PIL (T *Lane 4*, *circled*; *upper arrow*, position on gel). This result is consistent with the two PIL foci being derived from distinct epithelial clones. There is loss or diminution of the wild-type sequence from these samples, consistent with loss of the second *p16* allele from these lesions.

of both *p16* and K-*ras* mutations in a PIL was not accompanied by the histological characteristics that typically accompany "advanced" or "aggressive" neoplasms. Although one must be cautious in interpreting the results from a single instance, an interesting possibility is suggested by this finding: histological characteristics do not always correlate with advanced neoplastic precursor lesions; hence, molecular analysis may prove to be a more sensitive assay for the identification of the more clinically relevant precursor lesions of the infiltrating carcinomas.

The work described here represents an initial step toward a full understanding of the sequence of genetic events that occur in pancreatic tumorigenesis. As the technology for microdissection and molecular genetic analysis improves, the type of analysis detailed here can be expanded to include other genes and assays to further illuminate the sequence and clinical implications of mutational events that take place in intraductal pancreatic epithelium.

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