

KRAS2 Mutations in Human Pancreatic Acinar-Ductal Metaplastic Lesions Are Limited to Those with PanIN: Implications for the Human Pancreatic Cancer Cell of Origin

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Abstract

Pancreatic intraepithelial neoplasia (PanIN) is a precursor to invasive ductal adenocarcinoma of the pancreas. Observations made in genetically engineered mouse models suggest that the acinar/centroacinar compartment can give rise to ductal neoplasia. To integrate findings in mice and men, we examined human acinar cells, acinar-ductal metaplasia (ADM) lesions, and PanINs for *KRAS2* gene mutations. Surgically resected pancreata were screened for foci of ADM with or without an associated PanIN lesion. Stromal cells, acinar cells, ADMs, and PanINs were separately isolated using laser capture microdissection. *KRAS2* status was analyzed using genomic DNA isolated from the microdissected tissue. Twelve of these 31 foci of ADM occurred in isolation, whereas 19 were in the same lobules as a PanIN lesion. All 31 microdissected foci of acinar cells were *KRAS2* gene wild-type, as were all 12 isolated ADM lesions lacking an associated PanIN. *KRAS2* gene mutations were present in 14 of 19 (74%) PanIN lesions and in 12 of the 19 (63%) foci of ADM associated with these PanINs. All ADM lesions with a *KRAS2* gene mutation harbored the identical *KRAS2* gene mutation found in their associated PanIN lesions. Ductal neoplasms of the human pancreas, as defined by *KRAS2* gene mutations, do not appear to arise from acinar cells. Isolated ADM lesions are genetically distinct from those associated with PanINs, and the latter may represent retrograde extension of the neoplastic PanIN cells or less likely are precursors to PanIN. (Mol Cancer Res 2009;7(2):230–6)

Introduction

Pancreatic intraepithelial neoplasia (PanIN) has been recognized morphologically for over a century, but the biological significance of these lesions has only recently been defined (1-3). In 1976, Cubilla and Fitzgerald described histologically distinct proliferative lesions in the pancreatic ducts and ductules adjacent to infiltrating exocrine pancreatic cancer. They showed that these lesions were more common in pancreata with an invasive carcinoma than those without carcinoma (2). PanINs are currently classified into three grades, PanIN-1, PanIN-2, and PanIN-3, based on the degree of architectural and cytologic atypia (3). Several histopathologic and clinical studies have provided strong evidence that PanINs in the pancreas can progress to invasive carcinoma (4). In addition, molecular genetic analyses have shown that almost all of the genetic alterations identified in infiltrating ductal adenocarcinomas of the pancreas can also be identified in PanINs, and the prevalence of these genetic alterations in PanINs increases in parallel with morphologic progression from PanIN-1 to PanIN-3 to invasive carcinoma (3). Based on these studies, a genetic progression model has been established for PanINs, and PanINs are now recognized as one of the precursors to invasive adenocarcinoma of the pancreas (5). In addition, these studies have shown that *KRAS2* gene mutations are one of earliest genetic alterations in pancreatic neoplasia; ~45% of early PanIN lesions and 90% of infiltrating pancreatic ductal adenocarcinomas harbor *KRAS2* gene mutations (6-11). Therefore, *KRAS2* gene mutations provide a tool to study the origins of human pancreatic neoplasia.

Several animal models have recently been generated, which recapitulate the morphologic progression of human pancreatic ductal adenocarcinoma, and these models have been used to define the populations of cells that can give rise to invasive pancreatic adenocarcinoma (12). Not surprisingly, most of these models are driven by mutant *KRAS* (13, 14). The expression of mutant *KRAS* in ductal epithelium under the control of cytokeratin 19 promoter failed to produce PanINs or pancreatic ductal adenocarcinoma (15); however, selective expression of endogenous mutant *KRAS* in acinar/centroacinar cells during early embryonic development produces a full range of mouse PanINs (mPanIN) and invasive pancreatic ductal adenocarcinoma (16-18). In addition, acinar-ductal metaplasia (ADM) is a prominent component of many of these genetically engineered mouse models (17-19), and in some models, ADM appears to

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precede the appearance of mPanIN lesions (17). The genetically engineered mouse models, therefore, suggest the possibility of an acinar/centroacinar origin of pancreatic adenocarcinoma, with progression to ADM, mPanINs, and eventually invasive carcinoma.

In humans, Brune et al. (20) and Detlefsen et al. (21) have observed that PanIN lesions are frequently associated with lobulocentric atrophy and ADM. In addition, it has been shown that these ADM structures contain both acinar and duct cell phenotypes (19). These observations in human tissues, when taken together with the findings in genetically engineered mouse models, suggest the possibility that human “ductal” neoplasia may arise before the PanIN lesion, perhaps even in acinar cells. (22). To integrate findings in mice and men, we examined human acinar cells, ADM lesions, and PanINs for *KRAS2* gene mutations to determine if *KRAS2* gene mutations occur before the development of PanINs in human pancreata.

Results

KRAS2 Gene Status in Acinar Cells

To address these questions, *KRAS2* gene mutations were analyzed because these mutations are one of the earliest known mutations to occur in human PanINs and because most of the genetically engineered mouse models that have suggested an acinar cell origin of pancreatic cancer are driven by mutant *KRAS*. Thirty-one foci of acinar cells associated with an ADM and/or a PanIN lesion were sequenced and all 31 were *KRAS2* gene wild-type both by standard sequencing and by LigAmp

(Table 1). The LigAmp technique is an ultrasensitive point mutation detection strategy capable of detecting low levels of point mutant DNA in a dominant population of wild-type molecules (1:10,000; refs. 23, 24). This finding suggests that acinar cells in human pancreata are unlikely “the cell of origin” for human pancreatic ductal adenocarcinoma at least as defined by a clonal *KRAS2* gene mutation. Similarly, all 31 foci of microdissected stromal cells were *KRAS2* gene wild-type by both techniques.

Initial Characterization of ADM Lesions

ADM is characterized by the abnormal transformation of a mature acinar cell to a cell with ductal differentiation (25). ADM lesions initially appear as collections of acinar cells, which have lost some of their normal apical granularity and polarization. These cells then gradually acquire features of ductal differentiation as they become cuboidal to columnar and express mucin (Fig. 1). These metaplastic cells may focally involve a lobule, or a lobule of acinar cells may be completely replaced by cells with ductal differentiation. At the interface between foci of complete metaplasia and normal acini, the ADM structures may contain both cells with acinar differentiation and cells with ductal differentiation (Fig. 1E and F).

In this study, foci of ADM associated with a PanIN lesion were analyzed separately from foci of ADM not associated with a PanIN lesion. In the latter cases, the ducts draining the metaplastic lobules were histologically normal, and the metaplasia lesions typically contained little or no mucin (Fig. 1B,

Table 1. *KRAS2* Mutations in Acinar Ductal Metaplasia with (Group I) or without Associated PanINs (Group II)

Group	Case no.	Age	Sex	Diagnosis	Stroma	Acinar cells	Metaplasia	PanIN	
Group I (lesions with an associated PanIN)	IA	MP1	80's	M	Endocrine neoplasm	WT	WT	GAT	GAT
		MP4	50's	F	Endocrine neoplasm	WT	WT	GTT*	GTT
		MP5	80's	F	Cholangiocarcinoma	WT	WT	GAT	GAT
		MP6	60's	F	Ampullary adenocarcinoma	WT	WT	GAT*	GAT*
		MP8	50's	M	Chronic pancreatitis	WT	WT	GTT*	GTT
		MP9	70's	M	Duodenal adenocarcinoma	WT	WT	GTT*	GTT*
		MP11	60's	F	Duodenal adenocarcinoma	WT	WT	GTT	GTT
		MP13	40's	F	Endocrine neoplasm	WT	WT	GAT	GAT
		MP14	70's	F	IPMN	WT	WT	GAT*	GAT
		MP16	40's	F	Serous cystadenoma	WT	WT	GAT + GTT	GTT
	MP17	30's	F	Endocrine neoplasm	WT	WT	GTT*	GTT*	
	MP19	50's	M	Endocrine neoplasm	WT	WT	GTT*	GTT	
	IB	MP3	60's	M	Ductal adenocarcinoma	WT	WT	WT	GTT*
		MP15	60's	F	Ductal adenocarcinoma	WT	WT	WT	CGT
	IC	MP2	80's	M	Ductal adenocarcinoma	WT	WT	WT	WT
		MP7	60's	F	Ampullary adenocarcinoma	WT	WT	WT	WT
		MP10	60's	F	Ampullary adenocarcinoma	WT	WT	WT	WT
		MP12	60's	M	Serous cystadenoma	WT	WT	WT	WT
	Group II (lesions without an associated PanIN)	M	MP18	60's	M	Ampullary adenocarcinoma	WT	WT	WT
M1			60's	M	Chronic pancreatitis	WT	WT	WT	WT
M2			60's	M	Ductal adenocarcinoma	WT	WT	WT	WT
M3			50's	M	Serous cystadenoma	WT	WT	WT	WT
M4			50's	M	Chronic pancreatitis	WT	WT	WT	WT
M5			60's	M	Chronic pancreatitis	WT	WT	WT	WT
M6			60's	M	Chronic pancreatitis	WT	WT	WT	WT
M7			60's	M	Chronic pancreatitis	WT	WT	WT	WT
M8			70's	M	Serous cystadenoma	WT	WT	WT	WT
M9			50's	F	Serous cystadenoma	WT	WT	WT	WT
M10			30's	F	Endocrine neoplasm	WT	WT	WT	WT
M11			60's	M	Ampullary adenocarcinoma	WT	WT	WT	WT
M12	60's	M	IPMN	WT	WT	WT	WT		

NOTE: Case numbers and mutations are shown in bold. WT, wild-type GGT codons. Age is provided as decades of life to maintain patient privacy. Asterisk, cases that were wild-type by DNA sequencing but where a mutation was found using LigAmp. Abbreviation: IPMN, intraductal papillary mucinous neoplasm.

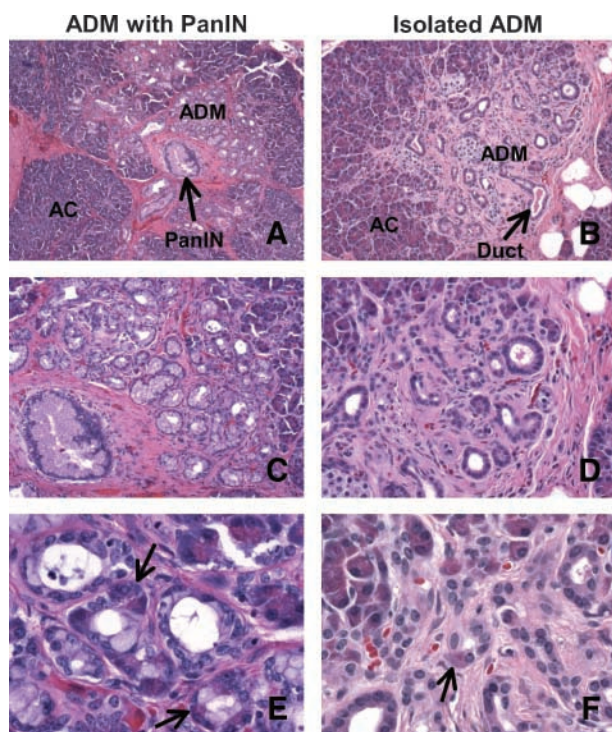


FIGURE 1. ADM with (A, C, and E) and without (B, D, and F) PanINs. **A.** H&E staining ($\times 40$) showing central PanIN lesion, ADM, and acinar cells (AC) in an acinar lobule. **B.** H&E staining ($\times 40$) showing ADM-only lesions that are composed of normal duct, metaplastic acinar structures (ADM), and normal acinar cells. **C.** Higher resolution ($\times 100$) of the ADM showing that metaplastic cells contain abundant intracytoplasm mucin, resembling ductal epithelium in the PanIN. **D.** Higher magnification ($\times 100$) of the ADM showing that metaplastic cells are cuboidal cells with minimal mucin. **E.** Higher resolution ($\times 200$) of the interface between ADM and normal acinar cells. Note that some metaplastic structures are composed of both acinar cells (arrows) and duct-like cells. **F.** Higher resolution ($\times 200$) of the interface between ADM and normal acinar cells. Note that some metaplastic structures are composed of both acinar cells (arrows) and duct-like cells. Arrows, residual acinar cells in partially metaplastic acini (E and F).

D, and F). Once we identified an isolated ADM lesion, we cut and stained additional 12 sections ($\sim 60 \mu\text{m}$) for laser capture microdissection. These confirmed the absence of coexisting PanIN in the planes of section examined, although we cannot exclude the possibility of coexisting PanIN in other regions not examined.

To test whether the two types of ADMs varied in proliferation rates, we performed immunohistochemistry for Ki-67, which showed that both ADM lesions (with and without an associated PanIN lesion) have low proliferative indices; however, ADMs associated with a PanIN have a slightly higher proliferation rate (2.8% versus 1.6%; $P = 0.047$). To assess if either lesion had high rates of apoptosis, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining, which was essentially negative in both lesions, with only extremely rare positive cells.

KRAS2 Status in Isolated ADM Lesions

Twelve ADM lesions involving lobules without an associated PanIN in the same lobule were examined (Table 1, group II,

M1-M12). The metaplastic acinar cells in the cases selected were small, cuboidal duct-like epithelium with no mucinous cytoplasm (see Fig. 1B, D, and F and Materials and Methods). The 12 lesions were selected from 9 surgically resected pancreata. Neither PanIN lesions nor pancreatic invasive carcinomas were present in the same slides to avoid contamination by these lesions/cancers.

Sequencing the *KRAS2* gene in these 12 lesions revealed that all 12 were *KRAS2* wild-type. No mutations were found in the pure ADMs. As expected, the acinar and stromal cells in these cases were also *KRAS2* gene wild-type. Testing the pure ADM lesions for the possibility of small numbers of mutation containing cells using LigAmp confirmed that they were *KRAS2* gene wild-type.

KRAS2 Gene Status in ADM Lesions Associated with PanINs

Nineteen ADM lesions associated with a PanIN lesion were microdissected from 18 surgically resected pancreata (designated as MP1-MP19 in Table 1). The nonneoplastic stromal cells, histologically normal acinar cells, foci of ADM, and PanIN lesions from each of these 19 foci were separately microdissected (Fig. 2) and then analyzed for activating *KRAS2* gene mutations by DNA sequencing and by LigAmp. Codon 12 *KRAS2* gene mutations were detected in 14 of 19 (74%) PanIN lesions (Table 1, groups IA and IB). These mutations include GGT to GTT (8 of 14), GAT (5 of 14), and CGT (1 of 14). Ten of the 14 *KRAS2* gene mutations were detected by DNA sequencing and an additional 4 only by the more sensitive technique LigAmp (samples MP6, MP9, MP17, and MP3; designated with an asterisk in Table 1), suggesting that the latter 4 PanINs harbored lower-level *KRAS2* gene mutations.

In 12 of the 14 (86%) lesions in which a *KRAS2* gene mutation was identified in the PanIN, a *KRAS2* gene mutation

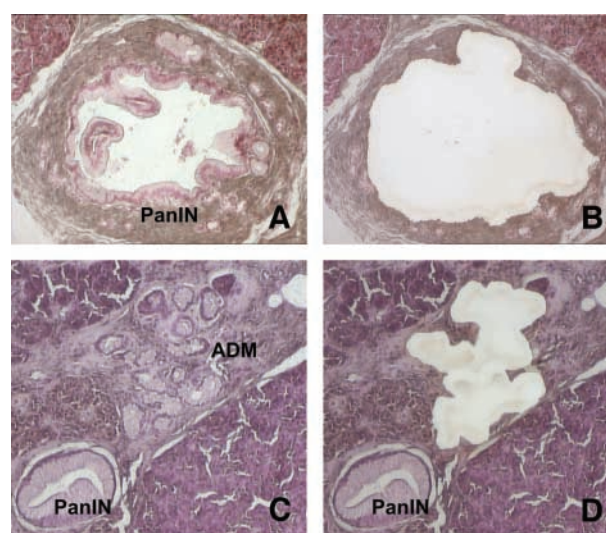


FIGURE 2. Representative photographs taken before and after microdissection of ADM lesions with associated PanINs. Photographs before (A) and after (B) microdissection of the PanIN. Representative photographs taken before (C) and after (D) microdissection of the ADM in MP16. Note that dissection of the ADM was done first to prevent potential contamination by the PanIN.

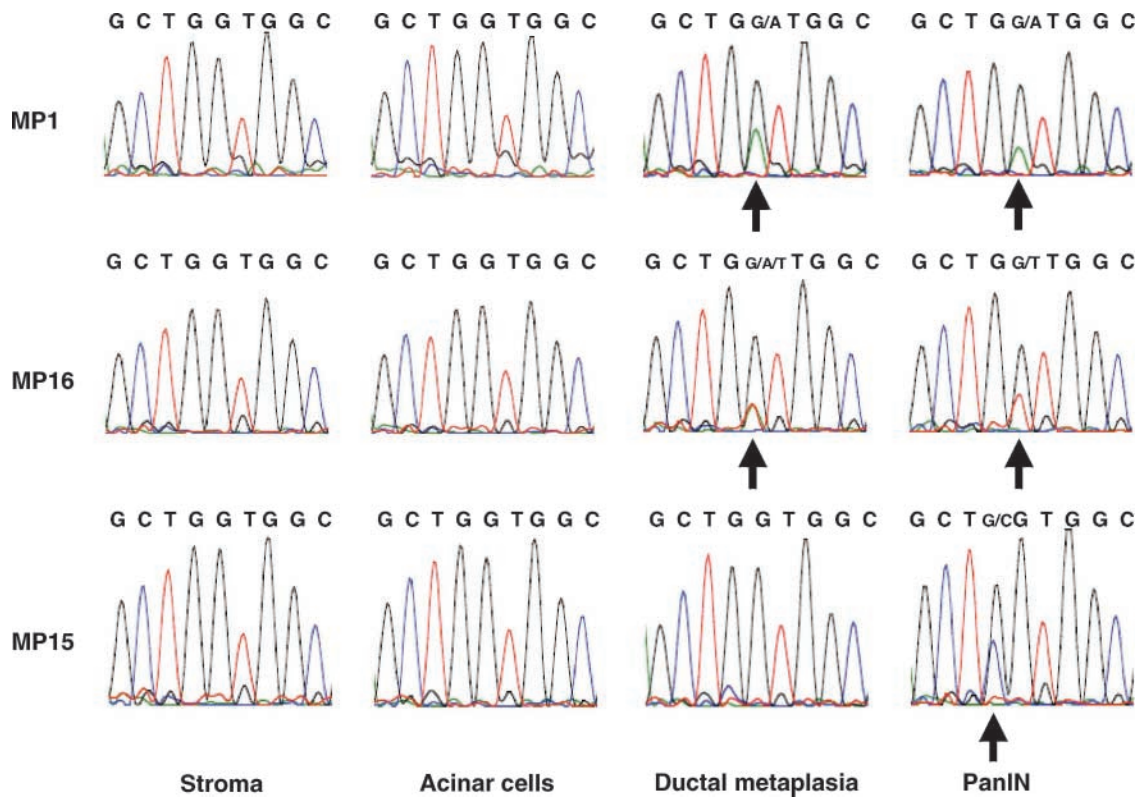


FIGURE 3. Representative *KRAS2* gene sequences, from left to right, in the stroma cells, acinar cells, ADM, and PanINs. Top row, representative *KRAS2* gene sequences from MP1. Note that the same *KRAS2* gene mutation (arrows) is present in both ADM and PanIN lesions. Middle row, representative *KRAS2* gene sequences from MP16. Note that two *KRAS2* gene mutations (GAT and GTT; arrows) are present in the ADM, whereas only one mutation (GTT) is present in the PanIN. Bottom row, representative *KRAS2* gene sequences from MP15. Note that the *KRAS2* gene mutation (CGT; arrow) is only present in the PanIN.

was also found in the associated ADM lesion, and in all 12 of these cases, the same mutation was found in the PanIN lesion and in the associated focus of ADM (group IA). These 12 mutations included GGT to GAT (5 cases), GGT to GTT (6 cases), and GGT to GAT/GTT (1 case). Representative *KRAS2* sequences of lesion MP1 from the stroma, acinar cells, ADM, and PanIN lesions are shown in Fig. 3. Cases were reviewed before microdissection by at least two of us (C.S., S-M.H., and R.H.H.) and photographed after microdissection to rule out cross-contamination between the microdissected PanIN lesions and foci of ADM (Fig. 2). In addition, for each set, special precautions were taken during microdissection; for example, samples were always collected in a sequence of the stroma cells, acinar cells, ductal metaplasia, and PanIN to minimize the potential for cross-contamination. Interestingly, in lesion MP16, a second mutation (GAT) was found in the associated ADM in addition to the GTT mutation identified in the PanIN lesion (Fig. 3).

In the remaining 2 of the 14 PanINs in which a *KRAS2* gene mutation was found in a PanIN, the associated foci of ADM were *KRAS2* wild-type (Table 1, group IB). The *KRAS2* gene mutations in these 2 PanINs included GGT to GTT (1 of 2) and CGT (1 of 2). Representative *KRAS2* DNA sequences from lesion MP15 are shown in Fig. 3. In this case, a GGT to CGT mutation was identified in the PanIN. However, no mutations were detected in either the accompanying ADM or adjacent acinar and stromal cells.

The remaining 5 cases with a PanIN lesion (Table 1, group IC) showed no *KRAS2* gene mutations in either the PanINs or the acinar-ductal metaplastic lesions.

Discussion

An understanding of the earliest precursors to invasive pancreatic cancer can be built by combining morphologic observations with careful molecular genetic studies. Such analyses, for example, have shown that PanIN lesions are a precursor to invasive ductal adenocarcinoma of the human pancreas (5). Genetically engineered mouse models in which genetic alterations are specifically targeted to selected cell populations can also be used to identify the cells, which when genetically manipulated, can give rise to a cancer (13, 14, 16-18). It is important that these two approaches to identifying precursors to invasive cancer are periodically reconciled such that the studies of human disease can benefit from insights gained in mouse models and so that those studying mouse models can develop models that most accurately reflect the human condition (26).

The findings from several genetically engineered mouse models of pancreatic neoplasia have recently suggested that the acinar cell compartment in the pancreas can give rise to neoplasms with a ductal phenotype (16-18). For example, in one model, the expression of an oncogenic *KRAS* transgene in acinar cells induced occasional low-grade PanINs (27), suggesting that

acinar cells could be “cell of origin” of pancreatic ductal carcinoma. More recently, Zhu et al. observed that ADM was the earliest prominent change to occur in LSL-*KRAS*^{G12D/+}/*p*^{48Cre+} mice, where metaplastic cells repressed acinar cell features and activated expression of the duct cell genes (18). They also showed that ADM and early mPanIN lesions in their mouse model exhibited comparable cellular and molecular properties. Similarly, another group has shown that selective expression of an endogenous mutant *KRAS* oncogene in embryonic cells of acinar/centroacinar lineage resulted in a full spectrum of mPanIN-1 to mPanIN-3 and invasive carcinoma (17). In contrast, selective expression of oncogenic *KRAS2* transgene in ductal lineages driven by the cytokeratin 19 promoter failed to induce mPanINs or invasive carcinoma (15). These observations made in genetically engineered mouse models have led to the hypothesis that the acinar/centroacinar compartment and not ductal cells give rise to mouse ductal neoplasia.

These results from the mouse models have led to a reevaluation of the early morphologic changes in human pancreatic ductal neoplasia. For example, it is now recognized that human PanIN lesions are often associated with foci of ADM. Detlefsen et al. documented a close association between lobular fibrosis and PanINs in elderly patients (21). The fibrotic foci shown in their report included ADM in addition to acinar atrophy and fibrosis. Similarly, Brune et al. examined PanIN lesions and parenchymal changes in the pancreas in patients having a strong family history of pancreatic cancer and found a close association of PanINs, even PanIN-1A, with atrophy of the lobular unit surrounding the duct containing the PanIN (20). Atrophic lobules exhibited a thinning of the acinar cells, loss of the apical granular cytoplasm, and slight dilatation of the acinar lumen, that is, ADM. Although these investigators hypothesized that the PanINs developed first, producing small ductal obstructions, in turn, progress to lobulocentric atrophy and ADM. These same observations, interpreted in light of the observations made in the genetically engineered mouse models, could be interpreted as suggesting that ADM precedes the development of human PanIN lesions. Indeed, in the present study, we observed that some foci of ADM arise in the absence of PanIN lesions, in areas where the associated pancreatic duct appears morphologically normal.

In the current study, we integrated the findings made in genetically engineered mouse models with the genetic changes in human acinar cells, foci of human ADM, and human PanINs. In all of the cases we examined, the acinar cells surrounding ADM lesions were *KRAS2* gene wild-type. This finding suggests that acinar cells in human pancreata are unlikely “the cell of origin” for human pancreatic ductal adenocarcinoma at least as defined by a clonal *KRAS2* gene mutation. By contrast, three-fourths of the PanIN lesions harbored an activating point mutation in the *KRAS2* gene, and these mutations were of the type seen in infiltrating ductal adenocarcinomas of the pancreas, consistent with the hypothesis that PanINs are precursor lesions that can progress to infiltrating adenocarcinoma (6). Of mutation bearing PanINs, 85% of their associated ADM lesions harbored a *KRAS2* gene mutation. In contrast, when these lesions occurred in isolation, without an associated PanIN lesion, they were always *KRAS2* wild-type. The only time we observed a *KRAS2* gene mutation

in a lesion of ADM was when the lesion was associated with a PanIN, and in all instances, the same mutation was found in both the PanIN and the lesion of ADM. The failure to find a *KRAS2* gene mutation in an ADM lesion not associated with a PanIN, combined with the finding that the two lesions harbor the same mutation, suggests that the ADM lesions associated with a PanIN may represent retrograde extension of the PanIN into the smaller ducts. It is also possible that there are two distinct molecular mechanisms of ADM formation, one that occurs in isolation, has low mucin content, and rarely involves mutations in *KRAS2*. The second ADM mechanism occurs within the same lobule as PanIN, contains mucin, and generally bears the same *KRAS2* gene mutation as the PanIN.

Our failure to show *KRAS2* gene mutations in isolated ADM lesions suggests that a centroacinar origin for human pancreatic cancer is less likely; however, we cannot rule out this possibility. Studies of genetically engineered mouse models have shown that centroacinar cells can give rise to pancreatic neoplasia, particularly after injury promotes differentiation into ductal cells. Most of the promoters used in the genetically engineered mouse models are expressed in centroacinar cells, and findings in mice with targeted *PTEN* gene deletion suggest that ductal metaplasia results from the expansion of centroacinar cells rather than transdifferentiation of acinar cells (28).

Some conclusions can be made about the relationship between PanINs, their associated ADMs and *KRAS2* gene mutations. First, although *KRAS2* gene mutations occur commonly in PanIN (74%), mutation of this gene appears not to be essential for PanIN formation, as mutations were absent in 26% of lesions. Alternatively, activation of the *KRAS2* pathway may be essential, but the pathway activation may be occurring through a less common *KRAS2* mutation (e.g., codon 61) or a currently unidentified alternative pathway member in *KRAS2* wild-type cases (11). Second, when a PanIN harbors a *KRAS2* gene mutation, the vast majority of ADMs within the same lobule will bear the same mutation, suggesting that the two lesions derive from one another. The question of “who is the mother and who is the daughter?” cannot be definitively determined from these data. The occurrence of *KRAS2* gene mutations detected in the PanIN-associated metaplastic acinar structures may be due to retrograde extension of the PanIN cells into the smaller ducts associated with acini, arising from physical backward extension of PanIN cells into smaller ducts producing the ADM phenotype. It is also alternatively conceivable that ADMs are the initial lesion and that those with *KRAS2* mutations tend to progress to PanIN. The latter possibility is supported by the observation that four of ADM mutations could only be detected using the more sensitive LigAmp assay and were missed by standard DNA sequencing, suggesting that the mutations may be present in only a subpopulation of cells within the ADM. This is further supported by the existence of two mutant *KRAS2*-containing PanINs where their associated ADMs were *KRAS2* wild-type. Alternatively, in the retrograde extension model, we would have to attribute this to incomplete extension or contamination of the microdissections with normal cells.

Although *KRAS2* gene mutations were not identified in isolated acinar cells or in isolated ADM lesions in this study, it is possible that this result was obtained because the isolated

lesions were early neoplastic lesions driven by something other than a *KRAS2* gene mutation. Although this is possible, the *KRAS2* gene was chosen for this study because it is the first known gene targeted in the development of human pancreatic neoplasia and because this is the very gene driving most of the genetically engineered mouse models that suggest the possibility of an acinar cell origin for pancreatic ductal neoplasia.

In summary, observations by several groups in several genetically engineered mouse models suggest that acinar cells, centroacinar cells, postulated stem cells, or ADM can be responsible for the development of pancreatic ductal neoplasia. Our current data, however, indicate that acinar cells are most likely not the origin of human pancreatic neoplasia (Fig. 4).

Materials and Methods

Case Selection

We obtained appropriate institutional approval for all experiments involving human subjects. All pancreatoduodenectomy (Whipple) resections in 2006 (~240 cases) were microscopically reviewed for the presence of either PanIN with associated ADM or ADM in isolation. Excluded were any cases that contained invasive carcinoma on the original slide or in the ~60 μm cut for microdissection. PanINs were identified as a microscopic papillary or flat noninvasive epithelial neoplasms arising in a pancreatic duct, composed of cuboidal to columnar cells with varying amounts of mucin and degrees of cytologic and architecture atypia (29). PanINs were further graded into PanIN-1, PanIN-2, and PanIN-3 lesions based on the degree of cytologic and architectural atypia (29). A similar classification system has been developed for precursor lesions in genetically engineered mouse models (12). Because we were interested in the earliest events in human pancreatic neoplasia, the PanIN lesions selected for analysis in this study were mostly PanIN-1 lesions, which are low-grade, flat, or papillary epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin (Fig. 1A and C).

Tissue Procurement and Processing

Twelve serial sections (5 μm) were cut from formalin-fixed, paraffin-embedded tissue blocks and placed on UV-irradiated membrane-coated slides (Carl Zeiss Microimaging). Slides were stained with H&E using a modified “H&E Staining for PALM Laser Capture” protocol. Briefly, the paraffin was first removed by semi-melting it at 65°C on a heat block for 1 min and then dipping it in xylene for 1 min. The slides were rehydrated with 100%, 96%, and 70% ethanol consecutively for 1 min each. Nuclei were stained with hematoxylin (Sigma-Aldrich) for 10 min, and the cytoplasm was stained with eosin (Sigma-Aldrich) for 5 min.

Stromal cells, mature acinar cells immediately adjacent to ADM, ADM lesions, and associated PanIN lesions within a single lobule were each separately microdissected in that order using a laser capture microdissection technique from PALM Technologies. Dissected tissues were catapulted into adhesive caps (Carl Zeiss Microimaging) by defocused laser pulses. Representative photographs before and after microdissection of the ADM and PanIN are shown in Fig. 2.

DNA Extraction and PCR Amplification of KRAS2

Genomic DNA was extracted from the microdissected tissues using QIAamp DNA Micro Kit from Qiagen. DNA concentrations were measured using Pico Green (Topac), ranging from 0.1 to 1 ng/μL. Isolated DNA (3 μL) was subjected to PCR amplification of the region of *KRAS2* gene containing codons 12 and 13. The forward and reverse primers were 5'-GAGTTTGTATTTAAAAGGTACTGGTGGGA-3' and 5'-TGGATCATATTCGTCCACAAAA-3', respectively. Amplifications were done by initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 min followed by 7 min final extension at 72°C using PCR SuperMix from Invitrogen. Due to low DNA concentrations for most of the specimens, a second round, 35-cycle nested PCR was followed using a nested PCR primer pair,

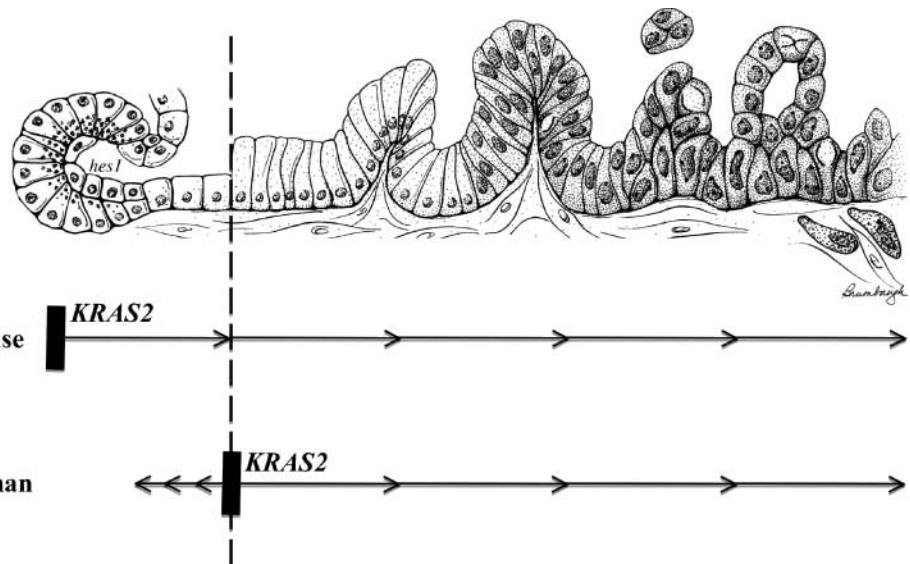


FIGURE 4. Potential “cell of origin” of pancreatic ductal neoplasia in mouse models and human pancreata. Adapted and reprinted, with permission, from ref. 5 (<http://www.annualreviews.org>). Vertical bars, cell of origin as currently established; backward arrows, remaining possibility that the PanIN precursor cell may originate in centroacinar cells.

5'-AAGGTACTGGTGGAGTATTTGATAGTG-3' (forward) and 5'-GATCATATTCGTCCACAAAATGA-3' (reverse). These PCR products were then purified using Qiagen PCR purification kit and sequenced using the BigDye Terminator 3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

LigAmp Analysis of KRAS2 Gene Mutations

LigAmp analyses for *KRAS2* gene mutations were also done to supplement the *KRAS2* gene sequencing with a significantly more sensitive method for detecting mutations. LigAmp analysis of *KRAS2* gene mutations has been described previously in detail (23, 24). To simultaneously determine mutant and wild-type *KRAS2*, we performed two reactions, each including a wild-type and one mutant (GAT or GTT) upstream oligonucleotide in addition to TaqMan probes for wild-type (*16S rDNA* probe) and mutant (*LacZ* probe) DNA in the reactions.

Immunohistochemical Labeling for Ki-67

Sections (5 μ m) were prepared from formalin-fixed, paraffin-embedded tissue blocks and immunolabeled for Ki-67 using a BenchMark XT automated stainer (Ventana) after steam-mediated antigen retrieval. Approximately 300 to 500 cells were counted depending on the size of the lesions. Percentages of positively labeled cells in the PanIN-associated and isolated ADM lesions were calculated and compared using Student's *t* test.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

Tissue sections (5 μ m) were first deparaffinized in fresh xylene and followed by rehydration in 95%, 85%, 75%, and 50% ethanol and by fixation in 4% paraformaldehyde. The sections were then washed twice with PBS, permeabilized with proteinase K, rinsed in PBS, and refixed with 4% paraformaldehyde. Apoptotic cells in the PanIN-associated and isolated ADM lesions were stained using The DeadEnd Colorimetric Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling System from Promega according to the manufacturer's protocol using mouse spleen and small intestine as positive and negative controls.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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