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Presence of Somatic Mutations in Most Early-Stage Pancreatic Intraepithelial Neoplasia

Mitsuro Kanda[*,1], **Hanno Matthaei**[*,1], **Jian Wu**[*,2], **Seung-Mo Hong**[1], **Jun Yu**[1], **Michael Borges**[1], **Ralph H. Hruban**[1,2], **Anirban Maitra**[1,2], **Kenneth Kinzler**[1,2], **Bert Vogelstein**[1,2], and **Michael Goggins**[1,2,3]

[1]Department of Pathology, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205-2196, U.S.A

[2]Department of Oncology, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205-2196, U.S.A

[3]Department of Medicine, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205-2196, U.S.A

Abstract

More information is needed about genetic factors that initiate development of pancreatic intraepithelial neoplasms (PanINs)—the most common precursors of pancreatic ductal adenocarcinoma. We show that >99% of the earliest stage, lowest-grade, PanIN-1 lesions contain mutations in *KRAS*, *p16/CDKN2A*, *GNAS*, or *BRAF*. These findings could improve our understanding the development and progression of these pre-malignant lesions.

Keywords

Pancreatic cancer; tumorigenesis; transformation; neoplasm

Pancreatic cancer is the 4th leading cause of cancer death in the USA[1]. PanINs are the most common precursor to invasive pancreatic adenocarcinoma[2]. They are microscopic lesions

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Correspondence and requests for reprints to: Michael Goggins, MD, Johns Hopkins Medical Institutions, Department of Pathology, 1550 Orleans Street, Baltimore, MD 21231. Tel: 410 955 3511, Fax: 410 614 0671, mgoggins@jhmi.edu.
*These authors contributed equally to this work.

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Author Contributions
Mitsuro Kanda; acquisition of data; analysis and interpretation of data; drafting of the manuscript
Hanno Matthaei: acquisition of data; analysis and interpretation of data; revision of the manuscript; obtained funding;
Jian Wu: acquisition of data; analysis and interpretation of data;
Seung-Mo Hong: acquisition of data;
Jun Yu: material support, acquisition of data;
Michael Borges: material support, acquisition of data;
Ralph Hruban: material support; acquisition and interpretation of data; revision of the manuscript
Anirban Maitra: material support; acquisition and interpretation of data; study supervision; revision of the manuscript
Ken Kinzler: study supervision; revision of the manuscript
Bert Vogelstein: study supervision; interpretation of data; revision of the manuscript: obtained funding;
Michael Goggins: study concept and design; study supervision; interpretation of data; drafting of the manuscript; obtained funding;

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(<5 mm diameter), and almost always too small to be identified by current imaging. Low-grade PanINs (PanIN-1) are common and their prevalence increases with age, while high-grade PanINs are uncommon and are usually found in pancreata with invasive pancreatic cancer. Multiple PanINs of all grades are frequently observed in individuals with inherited susceptibility to pancreatic cancer[3]. Over 90% of invasive invasive adenocarcinomas of the pancreas harbor oncogenic mutations in *KRAS* while *BRAF* mutations occur in a small subset of *KRAS*-wild-type pancreatic cancers[1,4]. Almost all invasive pancreatic cancers inactivate *p16/CDKN2A*. *GNAS* is mutated in ~60% of intraductal papillary mucinous neoplasms (IPMNs), and in some invasive pancreatic cancers arising in association with an IPMN[5]. Several genetic alterations identified in invasive pancreatic adenocarcinomas are also present in PanINs, with evidence of increasing prevalence of these alterations with PanIN grade[2]. However, the genes responsible for early PanIN development remain poorly understood. Thus, a meta-analysis evaluating studies of mutant *KRAS* prevalence in PanINs found that among patients with pancreatic ductal adenocarcinoma, *KRAS* mutations were detected by conventional methods in 36% of PanIN-1A, 44% of PanIN-1B, and 87% of high-grade PanIN lesions (PanIN-2 and -3)[6]. Data such as these indicate that *KRAS* mutations are more involved after PanIN initiation; genetic alterations that initiate tumorigenesis should have the same prevalence, independent of grade.

The goal of the current study was to employ more sensitive mutation detection methods to obtain a more detailed genetic understanding of early PanIN development. For this purpose, first we used laser capture to microdissect 169 PanINs (50 PanIN-1A, 52 PanIN-1B, 45 PanIN-2 and 22 PanIN-3 lesions) from 89 patients with benign and malignant pancreatic diseases (Supplementary Table 1), invasive pancreatic ductal adenocarcinomas from 12 patients, and normal pancreatic ducts from 20 patients. After DNA isolation and whole genome amplification, DNA was analyzed for somatic mutations in *KRAS*, *BRAF*, *GNAS*, and *p16/CDKN2A* using pyrosequencing and high-resolution melt-curve analysis. The limit of detection of these assays is ~5%, i.e. mutant alleles can be detected at concentrations of 5% or more (mutant: wild-type alleles: 1:20, cells: 1 in 10) (See Supplementary Methods).

Using pyrosequencing, *KRAS* codon 12 mutations were detected in 46 (92.0%) of 50 PanIN-1A, 48 (92.3%) of 52 PanIN-1B, 42 (93.3%) of 45 PanIN-2, and 21 (95.4%) of 22 PanIN-3 lesions (Supplementary Table 2). Occasional mutations of *KRAS* codon 13 and codon 61 were identified (Supplementary Table 2) and a 2nd non-dominant *KRAS* mutation was found in 6 of 169 PanINs. No evidence of *KRAS* amplification was found. Melt-curve analysis confirmed the presence of *KRAS* gene mutations in every sample that was positive by pyrosequencing (Supplementary Fig. 1). Overall, 163/169 (96.4%) of PanINs harbored *KRAS* mutations. No *KRAS* mutations were identified in normal pancreatic duct samples (Supplementary Table 2). Five of the 169 PanIN lesions tested by pyrosequencing had a 2nd minor KRAS codon 12 mutation. Many PanIN-1 lesions had low mutant *KRAS* concentrations (mean ~20% of alleles by pyrosequencing, Supplementary Table 3), perhaps explaining why prior studies reported a lower prevelance of *KRAS* mutations in low-grade PanINs. To check the purity of our laser capture microdissection, we repeated the microdissections from 16 of the PanINs, using another set of slides. The mutant *KRAS* concentrations in DNA from the second microdissection were not significantly different from those of the first microdissections (Supplemental methods). We also analyzed mutant *KRAS* concentrations in invasive pancreatic adenocarcinomas, and these samples had close to the concentrations of mutant *KRAS* one would expect if they consisted entirely of *KRAS*-mutant cancer cells without any contaminating wild-type (and presumably non-neoplastic) cells (mean 42.5% of *KRAS* alleles, not significantly different from the mean mutant *KRAS* allele concentration in PanIN-3 lesions). Indeed, we found that the average concentration of mutant *KRAS* alleles in PanINs increased significantly with increasing grade of PanIN (Supplementary Fig. 1C, Figure 2).

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These results indicate that virtually all PanINs harbor *KRAS* mutations. However, in the earliest PanIN lesions, these mutations are generally present in only a fraction of the cells comprising the lesion. The percentage of mutant *KRAS* cells in the PanIN progressively increases with the PanIN grade, consistent with a gradual expansion of the *KRAS*-mutant clone as the PanIN progresses.

We then sought to determine if mutations in other genes are present in the few *KRAS*-wild-type PanINs, particularly low-grade PanINs. As prior studies have found that *TP53* and *SMAD4* mutations do not appear until late in the neoplastic progression, we focused on *BRAF*, because it is sometimes mutant in *KRAS* wild-type cancers; *p16/CDKN2A*, because loss of *p16/CDKN2A* expression has been found in some early PanINs, and *GNAS*, because it is commonly mutated in another type of pre-malignant pancreatic lesion (IPMNs) [5].

Although *p16/CDKN2A* mutations were identified in only 17/147 of PanIN-1/2 lesions (11.5%), they were detected more often in *KRAS*-wild-type PanINs than in *KRAS*-mutant PanINs (P=0.0209). A similar trend was noted for *GNAS* mutations which were the only mutation identified in 2 PanINs (P=0.0886, Figure 2). Interestingly, similar to what was found for some IPMNs[5], among PanIN-1/2 lesions with both *GNAS* and *KRAS* mutations, mutant *GNAS* concentrations were higher than mutant *KRAS* (P=0.0084, paired t-test) (Supplementary Table 2), suggesting that low mutant *KRAS* concentrations in PanIN samples was not simply the result of contamination with DNA from nearby stromal cells. It also suggests that in some PanINs, the *KRAS* mutation arose later than the *GNAS* mutation. There were no histological differences in cell morphology within PanIN-1 lesions with low vs. high concentrations of either mutant *KRAS* or *GNAS* (Supplementary Figure 1). *GNAS* mutations were detected more often in PanINs from patients with a diagnosis other than pancreatic adenocarcinoma (P=0.0398). Overall, we were able to identify at least one mutation in *KRAS*, *GNAS p16/CDKN2A* or *BRAF*, in all but 1 of 169 PanINs (Supplementary Tables 1 and 2). No mutant *KRAS* or *GNAS* was detected in this one wild-type PanIN (Patient 72) even with the sensitive techniques we used (detection limit <1%).

To confirm the prevalence of *KRAS* and *GNAS* mutations in PanINs, we also conducted an independent analysis of an additional 37 PanIN lesions (11 PanIN-1, 20 PanIN-2, and 7 PanIN-3 lesions) using two additional ultra-sensitive technologies - Digital ligation (limit of detection 1/200 alleles) and BEAMing (limit of detection 1/1000 alleles)(Supplementary Methods) and found *KRAS* mutations in 94.6% of PanINs and *GNAS* mutations in 11.4% (Supplementary Table 4), with complete concordance of the mutation results with both platforms. A 2nd non-dominant *KRAS* mutation was found more often using these methods than by pyrosequencing, consistent with the lower limit of detection of these assays.

These results indicate that somatic mutations are required for the early development of virtually all PanINs. Our results are consistent with observations in genetically engineered mouse models in which mouse PanINs can be initiated by oncogenic *KRAS*[7]. Although low-grade PanIN cells have some metaplastic features, our results do not support the hypothesis that PanINs begin as metaplasias and only subsequently acquire genetic alterations. If this were true, more low-grade (early) PanINs would lack oncogenic mutations. (We found only one of 102 PanIN-1 lesions lacked a mutation). In prior studies, we have examined the metaplastic lesion known as acinar-to-ductal metaplasia (ADM) for evidence of genetic alterations (mutant *KRAS* and telomere length analysis) and did not find evidence from this analysis that ADMs are precursors to PanINs. The findings that many low-grade PanINs contain mixtures of mutant and wild-type *KRAS* cells, that *GNAS* mutation concentrations can be higher than *KRAS* mutation concentrations in the same PanIN, and that the average proportion of mutant *KRAS* within PanINs increases with PanIN grade, suggests that mutant *KRAS* alone provides only a modest selective advantage over neighboring cells. This finding

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suggests that the *KRAS*-mutant clone is partially restrained within the PanIN, possibly by oncogene-induced senescence[8,9] and this restraint is likely maintained until additional genetic and or epigenetic events (such as *p16/CDKN2A* inactivation) occur. The driving force behind the expansion of cells within PanINs that do not harbor mutant *KRAS* is not certain. One possibility is that PanIN-initiating event(s) precede oncogenic *KRAS* mutations. However, our genome[4] and methylome[10] studies indicate there are no other commonly mutated or epigenetically silenced[10] genes in pancreatic cancers that stand out as candidate initiators of PanIN development. Telomere shortening is observed in almost all low-grade PanINs[11] but this phenomenon could be a consequence of activation of oncogene stress-induced senescence programs[12] rather than an initiator of PanINs.

One unifying hypothesis to explain all these observations is that *KRAS*, and occasionally *p16/CDKN2A*, *GNAS*, or *BRAF*, mutations can initiate PanIN development, and that these mutant cells induce surrounding cells to proliferate. Such proliferation could come from autocrine and paracrine influences from *KRAS*-mutant PanIN cells, such as expression of Shh, and other developmental genes,[13,14] as well as stress-inducing signals that lead to senescence[15], and induce metaplastic features in PanIN epithelial cells including adjacent cells lacking *KRAS* mutations.

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References

1. Vincent A, et al. Lancet. 2011; 378:607–620. [PubMed: 21620466]
2. Hruban RH, et al. Clin Cancer Res. 2000; 6:2969–2972. [PubMed: 10955772]
3. Shi C, et al. Clin Cancer Res. 2009; 15:7737–7743. Epub. [PubMed: 19996207]
4. Jones S, et al. Science. 2008; 321:1801–1806. Epub 2008 Sep 1804. [PubMed: 18772397]
5. Wu J, et al. Sci Transl Med. 2011; 3:92ra66.
6. Lohr M, et al. Neoplasia. 2005; 7:17–23. [PubMed: 15720814]
7. Hingorani SR, et al. Cancer Cell. 2003; 4:437–450. [PubMed: 14706336]
8. Caldwell ME, et al. Oncogene. 2011 **epub aug 22**.
9. Lee KE, et al. Cancer Cell. 2010; 18:448–458. [PubMed: 21075310]
10. Vincent A, et al. Clin Cancer Res. 2011; 17:4341–4354. [PubMed: 21610144]
11. van Heek NT, et al. Am J Pathol. 2002; 161:1541–1547. [PubMed: 12414502]
12. Ben-Porath I, et al. J Clin Invest. 2004; 113:8–13. [PubMed: 14702100]
13. Strobel O, et al. Gastroenterology. 2010; 138:1166–1177. (epub dec1121 2009). [PubMed: 20026066]
14. Prasad NB, et al. Cancer Res. 2005; 65:1619–1626. [PubMed: 15753353]
15. Kuilman T, et al. Nat Rev Cancer. 2009; 9:81–94. [PubMed: 19132009]

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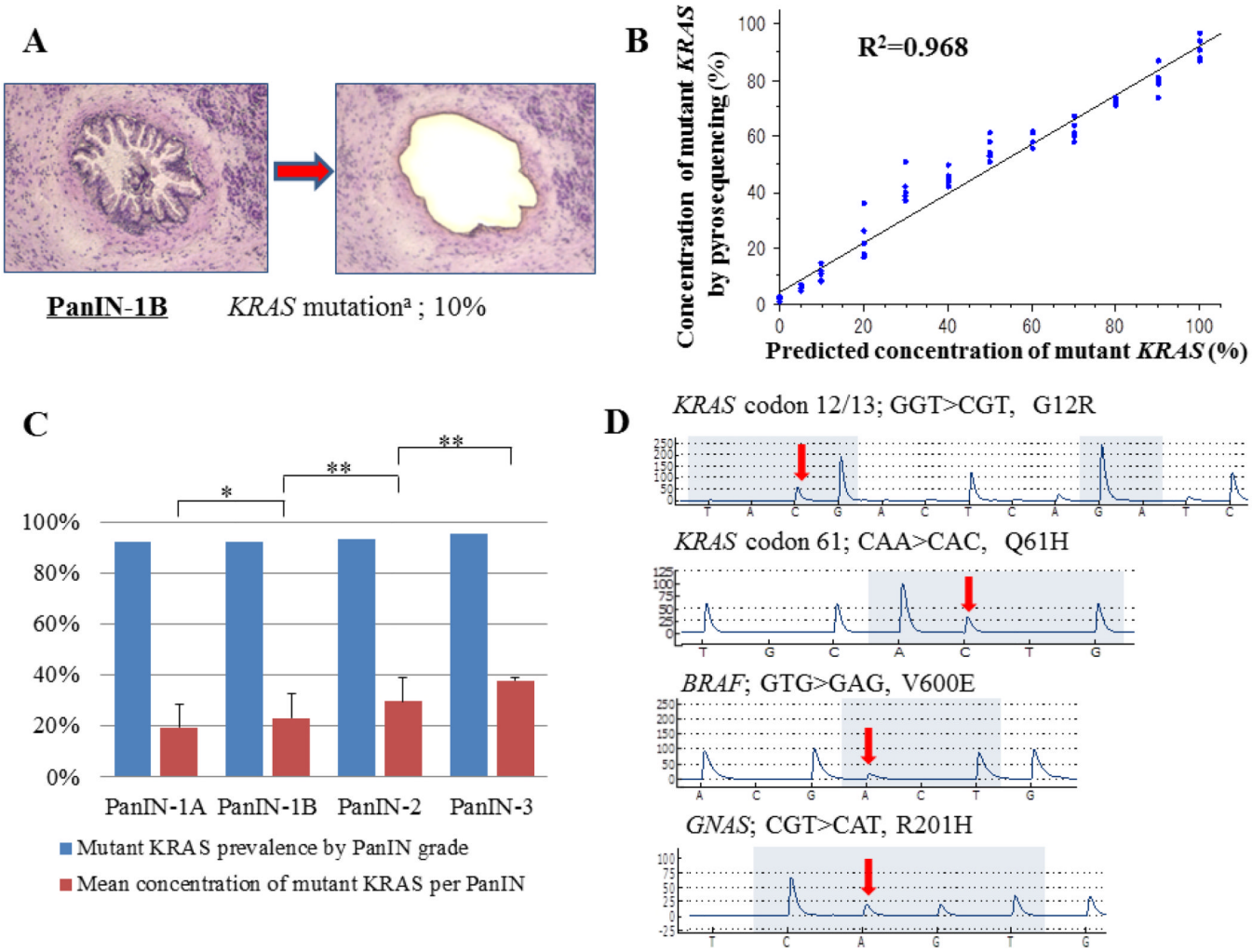

Figure 1.

(A) An example of a H + E stained PanIN before and after laser capture microdissection. Microdissection was performed at the duct epithelial cell borders to avoid contamination with stromal cells. (B) Scatter-plot graph of actual and predicted concentrations of mutant DNA by pyrosequencing. (C) Prevalence of *KRAS* codon 12 mutations and concentration of mutations per PanIN by grade of PanIN. *KRAS* codon 12 mutations were found in more than 92% of PanINs in every group. The average percentage of mutant *KRAS* alleles within a PanIN increased at each PanIN grade. (D) Representative pyrosequencing traces with mutant sequences highlighted by the arrows. * $P<0.05$, ** $P<0.001$. a = Concentrations of mutant DNA by pyrosequencing.

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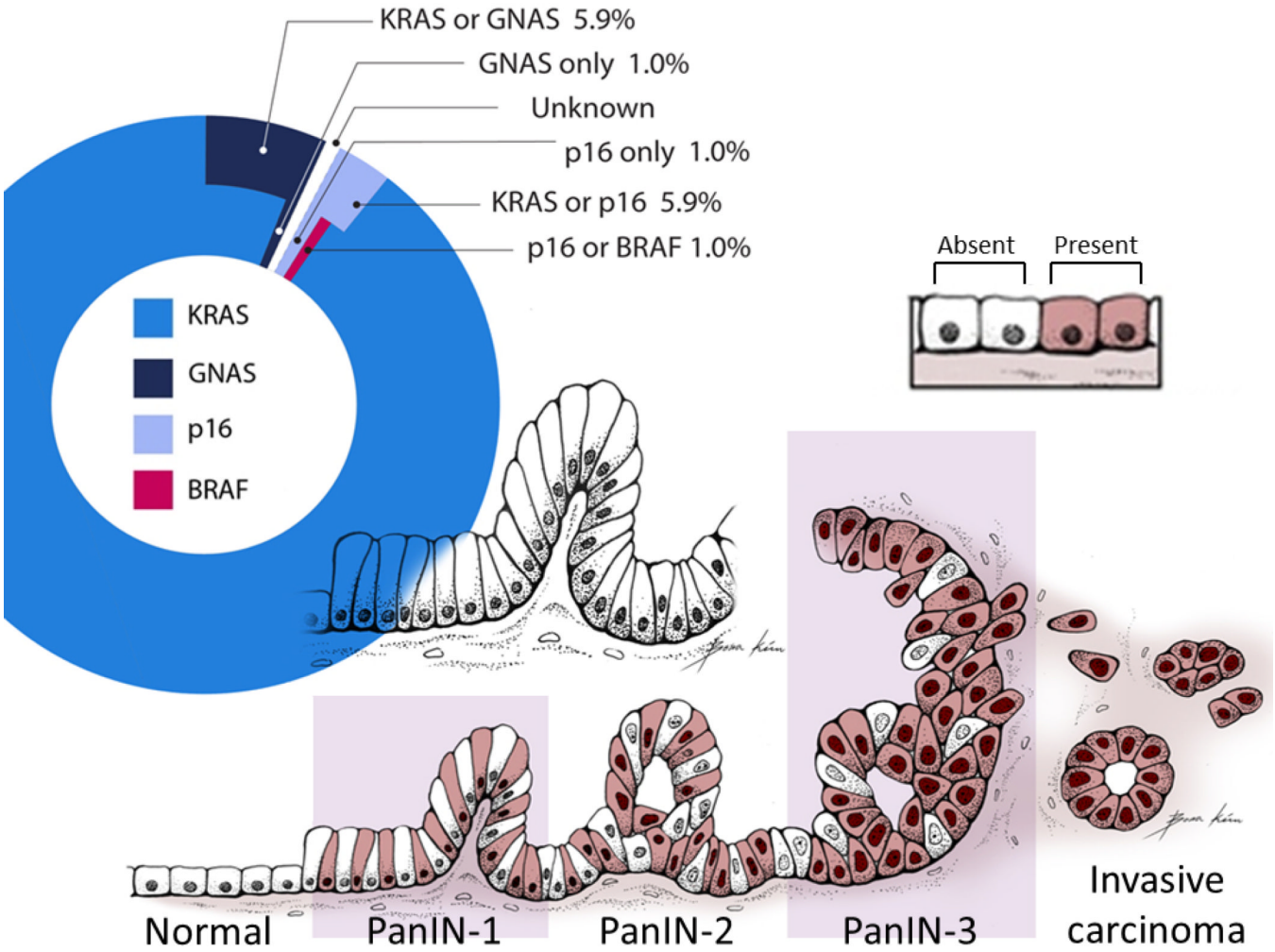

Figure 2. Initial mutations of PanIN-1 lesions

The pie chart in the upper portion of the figure is indicates the percentage of mutations in each of the genes tested (*KRAS*, *GNAS*, *p16*, *BRAF*) identified in PanIN-1 lesions. For PanIN-1 lesions with more than one mutation, we could not determine which gene was mutated first. For example, a few PanIN-1 lesions had both a *KRAS* mutation and a *GNAS* mutation so the initial mutation in these PanINs was indicated as arising in either "*KRAS* or *GNAS*".

The bottom portion of the figure is a schematic model illustrating the increasing percentage of mutant *KRAS* cells within PanIN lesions as they progress from a low-grade to a high-grade PanIN and to an invasive ductal adenocarcinoma, based on measurements of the average mutant *KRAS* concentrations per PanIN. Shaded cells (Present) represent PanIN cells with mutant *KRAS*, non-shaded cells (absent) represent PanIN cells without mutant *KRAS* (wild-type).

PanIN = pancreatic intraepithelial neoplasia.