Differential \textit{in vivo} tumorigenicity of diverse \textit{KRAS} mutations in vertebrate pancreas: A comprehensive survey

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Abstract

Somatic activation of the \textit{KRAS} proto-oncogene is evident in almost all pancreatic cancers, and appears to represent an initiating event. These mutations occur primarily at codon 12 and less frequently at codons 13 and 61. While some studies have suggested that different \textit{KRAS} mutations may have variable oncogenic properties, to date there has been no comprehensive functional comparison of multiple \textit{KRAS} mutations in an \textit{in vivo} vertebrate tumorigenesis system. We generated a Gal4/UAS-based zebrafish model of pancreatic tumorigenesis in which the pancreatic expression of UAS-regulated oncogenes is driven by a \textit{ptf1a:Gal4-VP16} driver line. This system allowed us to rapidly compare the ability of 12 different \textit{KRAS} mutations (G12A, G12C, G12D, G12F, G12R, G12S, G12V, G13C, G13D, Q61L, Q61R, and A146T) to drive pancreatic tumorigenesis \textit{in vivo}. Among fish injected with one of five \textit{KRAS} mutations reported in other tumor types but not in human pancreatic cancer, 2/79 (0.25\%) developed pancreatic tumors, with both tumors arising in fish injected with A146T. In contrast, among fish injected with one of seven \textit{KRAS} mutations known to occur in human pancreatic cancer, 22/106 (20.8\%) developed pancreatic cancer. All eight tumorigenic \textit{KRAS} mutations were associated with downstream MAPK/ERK pathway activation in preneoplastic pancreatic epithelium, while non-tumorigenic mutations were not. These results suggest that the spectrum of \textit{KRAS} mutations observed in human pancreatic cancer reflects selection based upon variable tumorigenic capacities, including the ability to activate MAPK/ERK signaling.
Introduction

The KRAS proto-oncogene is among the most frequently mutated genes in human tumors. To date, over 300 different KRAS mutations have been reported in human cancer (www.sanger.ac.uk/genetics/CGP/cosmic). Among these, base pair substitutions in codons 12, 13, 61 and 146 predominate, with different distributions observed in different tumor types. While these diverse mutations are often felt to have similar biologic significance, it remains to be seen whether they are all able to drive tumorigenesis in an equivalent manner. It is also unclear whether the different distribution of KRAS mutations observed in different human cancers reflects tissue-specific differences in mutation occurrence (as might result from differential carcinogen exposure), or to a variable capacity of specific mutations to confer a growth advantage in different tissues.

Among different tumor types, pancreatic cancer is characterized by especially high rates of KRAS mutation, with even early, pre-invasive lesions displaying KRAS mutation frequencies exceeding 90%\(^1\). To date, only two of these mutations (G12D and G12V) have been functionally evaluated in genetically engineered animal models of pancreatic neoplasia\(^2-^4\). While G12D and G12V are the two most common KRAS mutations observed in pancreatic cancer, up to 25% of pancreatic tumors will display other mutations, including G12C, G12R, G13D, Q61L and Q61R. Other tumor types, including colon cancer, display additional KRAS mutations, including G12A, G12F, G12S, G13C and A146T\(^1,^5\). The ability of many of these mutations to drive in vivo tumorigenesis has not yet been tested, reflecting the fact that our ability to detect somatic mutations has accelerated at a rate far beyond our ability to experimentally evaluate their functional implications.

As a means to accelerate the functional evaluation of somatic mutations identified in human cancer, the zebrafish has emerged as a promising model organism.\(^6,^7\) With respect to pancreatic tumorigenesis, stable transgenic zebrafish models of pancreatic cancer have been previously described.\(^8\) However, the time frame required to generate stable transgenic lines in zebrafish is not fundamentally different from that required in mice, meaning that this approach is not likely to meaningfully alleviate the discrepancy between pancreatic cancer gene discovery and in vivo functional evaluation of identified mutations. In order to address this issue, we generated a transient Gal4-VP16/UAS system for functionally evaluating candidate oncogenes in zebrafish pancreas. This has allowed us to effectively compare the ability of 12 different KRAS mutations (G12A, G12C, G12D, G12F, G12R, G12S, G12V, G13C, G13D, Q61L, Q61R, and A146T) to drive in vivo pancreatic tumorigenesis. In addition to providing insight regarding the varying capacities of different KRAS mutations to initiate pancreatic cancer, this system now provides a novel platform for the rapid functional annotation of additional somatic mutations identified in pancreatic cancer genomes.

Results and Discussion

Targeted expression of eGFP-KRAS\(^{mutant}\) transgenes in zebrafish pancreas

To functionally compare the ability of different human KRAS mutations to initiate pancreatic tumorigenesis, twelve different mutations (G12A, G12C, G12D, G12F, G12R,
G12S, G12V, G13C, G13D, Q61L, Q61R, and A146T) were selected for analysis. KRAS mutant alleles were generated by modifying a wild-type human KRAS cDNA using site-directed mutagenesis followed by full length sequencing to confirm successful mutation. Each mutant variant was expressed as an eGFP-KRASmutant fusion under the transcriptional control of a concatamerized 14xUAS element. Mosaic pancreatic expression was achieved by injection of UAS:eGFP-KRASmutant structures into hemizygous ptf1a:Gal4-VP16 transgenic embryos produced from a cross between the Tg(ptf1a:Gal4-VP16)JH16 BAC transgenic line and wildtype AB fish (Fig. 1A). Reflecting known patterns of ptf1a gene expression, eGFP expression was first observed in the developing hindbrain and cerebellum (Fig. 1B,C). Due to yolk autofluorescence, pancreas-specific expression of eGFP-KRASmutant transgenes proved to be difficult to detect in whole mount embryos (Fig. 1B and C, asterisk). However, confocal imaging of the micro-dissected pancreas from 5 dpf larvae revealed effective expression and membrane localization of the eGFP-KRASmutant protein (Fig. 1D-G). On day five, embryos showing eGFP fluorescence within the ptf1a expression domain were selected and raised to adulthood.

Relative tumorigenicity of eGFP-KRASmutant transgenes in zebrafish pancreas

At 3 months of age, fish were randomly selected for examination of eGFP fluorescence in the cerebellum and pancreas. All twelve versions of activated KRAS were associated with high frequencies of eGFP fluorescence in the cerebellum, as shown for G12D and G12V in Fig. 2A and I. The percentage of fish displaying cerebellar eGFP fluorescence is shown in Fig. 3 (light green bars), and ranged from 44%-100%. As in the case of previously reported ptf1a:eGFP-KRASG12V transgenic fish, no cerebellar or hindbrain tumors were observed in transgenic fish expressing UAS:eGFP-KRASmutant transgenes.

We next sacrificed adult fish with or without transcutaneous eGFP fluorescence in the cerebellum, and examined pancreatic transgene expression as assessed by eGFP fluorescence within dissected abdominal viscera. As in the case of cerebellum, all twelve versions of activated KRAS were associated with significant frequencies of pancreatic eGFP fluorescence, ranging from 8%-66.7%. Representative transcutaneous and pancreatic eGFP fluorescence for G12D and G12V are shown in Fig. 2B,C and Fig 2J,K, and additional examples of are shown in Supplemental Fig. S1. Rates of pancreatic eGFP fluorescence are depicted in Fig. 3 (dark green bars), and further immunohistochemical confirmation of eGFP-KRASmutant transgene expression is provided in Figure 4.

Dissected abdominal viscera were then subjected to detailed histological examination in order to determine the presence or absence of pancreatic tumor. The frequencies of pancreatic tumor formation for each version of activated KRAS are summarized in Fig. 3 (red bars). Eight out of twelve different KRAS mutations were associated with pancreatic tumor formation, typically at frequencies lower than those observed for pancreatic eGFP expression (G12C: 18.2%, G12D: 25%, G12R: 28.6%, G12V: 20%, G13D: 6.7%, Q61L: 20%, Q61R: 7.7%, A146T: 16.7%). In contrast, four out of twelve different KRAS mutations (G12A, G12F, G12S and G13C) failed to induce pancreatic tumor formation, even though they were expressed at frequencies equivalent to that observed for fully tumorigenic mutations, as determined by both gross and immunohistochemical examination of eGFP
fluorescence (Figures 3A and 4). No tumors were noted in control ptf1a:Gal4-VP16 transgenic fish not injected with eGFP-KRAS\textsuperscript{mutant} transgenes.

In comparing the ability of different activating KRAS mutations to drive tumorigenesis in zebrafish, we noted that all seven mutations previously reported in human pancreatic cancer (G12D, G12V, G12R, G12C, G13D, Q61L and Q61R) were effective in initiating pancreatic tumorigenesis. In contrast, among the five KRAS mutations reported in other tumor types but not in human pancreatic cancer (G12A, G12F, G12S, and G13C and A146T) only one (A146T) proved to be tumorigenic in zebrafish pancreas (Figure 3B). Cumulatively, 22/106 fish (20.8%) injected with one of the seven KRAS mutations observed in human pancreatic cancer developed pancreatic tumors, compared to 2/79 fish (0.25%) injected with one of the five KRAS mutations reported only in other tumor types. These data suggest that the different frequencies observed for different KRAS mutations in human pancreatic cancer likely reflect selection based upon variable tissue-specific tumorigenic capacities.

Characterization of pancreatic tumors and assessment of downstream signaling pathways

Representative tumor histologies are presented in Fig. 2 for G12D (Fig. 2D-H) and G12V (Fig. 2L-P), and histologies for all tumors are presented in Supplemental Fig. S1. Among the twenty-two tumors induced by the eight fully tumorigenic versions of oncogenic KRAS, there were no major differences in tumor histology. Each tumor displayed predominant features of acinar cell carcinoma, similar to that previously reported for ptf1a:eGFP-KRAS\textsuperscript{G12V} transgenic fish \textsuperscript{8}, and showed evidence of local tissue invasion and/or metastasis (Supplemental Fig. S2). All tumors also displayed widespread nuclear labeling for PCNA and phospho-ERK (Fig 4A-E and data not shown). In the case of G12V, G12C and G12R, ERK phosphorylation was evident even in residual normal acinar cells adjacent to primary tumors (Supplemental Fig. S3). In contrast, pancreatic tissue from fish injected with non-tumorigenic versions of KRAS showed no evidence of histologic abnormality and minimal to no labeling for these markers (Fig. 4F-I). This was true even in spite of widespread oncogene expression, as indicated by eGFP labeling.

Together, these results suggest that, at least in part, the differential frequencies of mutant KRAS alleles observed in human pancreatic cancer are reflective of corresponding differences in tumorigenic capacity. This is true in spite of the fact that known oncogenic RAS mutations are all thought to share a common mechanism of stabilizing active RAS:GTP complexes at the expense of inactive RAS:GDP. In the case of mutations in codons 12, 13 or 61, this is achieved through diminished intrinsic GTPase activity \textsuperscript{11}. Other mutations, including those in codons 116 and 119, are associated with a general decrease in RAS protein affinity for guanine nucleotides, shifting the equilibrium towards binding of more abundant GTP \textsuperscript{11}. Despite this unifying biochemical mechanism, RAS family members display highly divergent frequencies of mutation in different tumor types. Activating HRAS mutations promote bladder and salivary gland tumorigenesis, while NRAS mutations are associated with thyroid carcinoma, melanoma and myeloid malignancies\textsuperscript{12-15}. Oncogenic KRAS mutations, in contrast, are most frequently associated with endodermally-derived tumors, including pancreatic, colorectal and lung carcinomas\textsuperscript{5,16,17}. These tissue-specific differences in mutation frequency may reflect both cell lineage-specific differences in
KRAS, HRAS and NRAS gene expression, as well as unique subcellular localization/compartmentalization patterns associated with each family member. In addition, considerable evidence suggests that different mutant KRAS alleles may be associated with variable and highly context-dependent downstream effects. In NIH3T3 cells, codon 12 mutations produced stronger protection from apoptosis and enhancement of anchorage-dependent growth compared to codon 13 mutations, even though the two mutations were associated with similar levels of elevated downstream MAP kinase activity. Additional studies suggest that specific oncogenic KRAS alleles may confer unique chemosensitivity profiles and variable clinical outcomes.

Even more compelling evidence suggesting that individual KRAS mutant alleles may differ in their tumorigenic capacities is provided by data comparing the prevalence of individual KRAS mutations in tumor tissue compared to adjacent normal tissue. In normal human colonic epithelium, the prevalence of codon 12 and codon 13 mutations was found to be nearly equivalent, compared to a 14-fold excess of codon 12 mutations in associated cancers. Similar data are not available regarding the respective rates at which specific KRAS mutations arise in human pancreatic tissue. However, the current study suggests that, regardless of the rates of their initial appearance, different mutant KRAS alleles would be subject to a high degree of selection based on variable tumorigenic capacities.

While the current results clearly suggest KRAS allele-specific differences in the ability to induce pancreatic tumor formation, it might be argued that the different rates of tumor formation observed in our study may simply reflect differential expression levels. However, we think that this is unlikely to be a primary cause of differential tumorigenicity, as we employed eleven to twenty-five fish in each group to control for fish-to-fish variability in transgene expression, and even non-tumorigenic mutant KRAS alleles were associated with high rates of pancreatic eGFP fluorescence. In addition, based on the fact that our assay depends upon mosaic somatic expression, we are likely interrogating large numbers of individual transgene insertion sites, thereby controlling for transgene insertion site-specific influences leading to differential expression. Finally, the fact that those mutations previously identified in human pancreatic cancer were so much more tumorigenic than those not previously observed suggests that the current assay is unlikely to be confounded by arbitrary differences in transgene expression levels. Nevertheless, future initiatives may include comparison of candidate oncogenic mutations (either somatic or germline) expressed from an identical genomic locus, as might be achieved through either endonuclease-facilitated homologous recombination or phiC31-mediated targeted integration.

In summary, we have comprehensively surveyed the ability of twelve different oncogenic KRAS mutations to induce pancreatic tumors in a novel in vivo tumorigenesis assay. Our results suggest that the appearance or non-appearance of individual mutant KRAS alleles in human pancreatic cancer is highly associated with the tumorigenic capacity of these mutations in zebrafish. Similar zebrafish-based tumorigenic assays may be useful for in vivo functional interrogation of candidate oncogenic mutations identified in other human cancers.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Targeted expression of eGFP-KRAS\textsuperscript{mutant} transgene in zebrafish pancreas

(A) Schematic depiction of experimental design employing the Gal4-VP16/UAS system used to drive eGFP-KRAS\textsuperscript{mutant} transgene expression within the ptf1a expression domain.

(B-C) Lateral view of larval zebrafish (5dpf) under transmitted and fluorescent illumination, showing expression pattern of eGFP-KRAS\textsuperscript{mutant} transgene in the hindbrain. Due to yolk autofluorescence (asterisk *), pancreatic expression of the eGFP-KRAS\textsuperscript{mutant} transgene is difficult to detect in intact embryos. (D-G) Confocal image of microdissected pancreas from 5 dpf larval fish, revealing the membrane localization of eGFP-KRAS\textsuperscript{mutant} protein. Blue pseudocolor indicates DAPI labeling.
Fig. 2. Identification of Pancreatic Tumors induced by $\textit{KRAS}^{G12D}$ and $\textit{KRAS}^{G12V}$

(A and I) Transcutaneous fluorescence in the cerebellum (white arrow) from $\textit{KRAS}^{G12D}$ (A), and $\textit{KRAS}^{G12V}$ (I). (B and J) Transcutaneous fluorescence in the abdomen from $\textit{KRAS}^{G12D}$ (B), and $\textit{KRAS}^{G12V}$ (J). (C and K) Dissected abdominal viscera with an eGFP-positive tumor from $\textit{KRAS}^{G12D}$ (C) and $\textit{KRAS}^{G12V}$ (K). (D-H, L-P) Histological examination showed the pancreatic acinar tumors from $\textit{KRAS}^{G12D}$ and $\textit{KRAS}^{G12V}$. Frank acinar cell carcinoma is interspersed with areas of acinar cell hyperplasia. Boxed areas indicate regions depicted at higher magnification in adjacent images. Scale bars: 50 μm.
Fig. 3. Comparison of in vivo tumorigenic capacity among twelve different oncogenic KRAS\textsuperscript{mutant} alleles

(A) Percentage fish displaying eGFP fluorescence in the cerebellum (light green bars) and pancreas (darker green bars), along with incidence of pancreatic tumor formation at 3 months of age (red bars). Numbers in parentheses indicate number of fish examined for each mutant allele. (B) Comparison of KRAS\textsuperscript{mutant} allele-specific efficiency of tumor formation in zebrafish for human KRAS mutations previously observed in human pancreatic cancer (n=7) vs. those not previously observed (n=5). Tumor-forming efficiency is significantly greater among mutations previously reported in human pancreatic cancer (p<0.01 by T-test).
Fig. 4. Characterization of pancreatic tissue expressing tumorigenic and non-tumorigenic KRASmutant alleles

(A) Pancreatic tissue from uninjected control ptf1a:Gal4-VP16 fish had histologically normal pancreas and no evidence of tumor formation in any organ. Control pancreatic tissue also displayed no labeling for eGFP and minimal labeling for p-ERK and PCNA. (B-E) Representative pancreatic tissue from fish injected with tumorigenic mutations G12C, G12D, G12R and G12V. Identical results were also observed for fish injected with G13D, Q61L, Q61R, and A146T (data not shown). Resulting tumors were uniformly positive for eGFP and showed strong labeling for p-ERK and PCNA. (F-I) Representative pancreatic
tissue from fish injected with non-tumorigenic mutations G12A, G12F, G12S, and G13C. In spite of widespread expression of eGFP-KRASmutant transgenes, normal histology and minimal labeling for p-ERK and PCNA are observed. Regions outlined by dotted lines indicated areas depicted at higher magnification in adjacent images. Primary antibodies used for immunohistochemistry were rabbit anti-eGFP (Invitrogen, A11122, 1:400), rabbit anti-phospho-ERK (Cell Signaling Technology, 4370S, 1:400), and mouse anti-PCNA (DAKO, M0879, 1:400).