Critical role of oncogenic KRAS in pancreatic cancer (Review)

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Abstract. Pancreatic cancer is a human malignancy with one of the highest mortality rates and little progress has been achieved in its treatment in recent decades. Further improvement to the understanding of the biological and molecular mechanisms underlying the initiation and development of pancreatic ductal adenocarcinoma (PDAC) is required. Previous studies using genetically engineered mouse models have demonstrated that oncogenic GTPase KRas (KRAS) mutation is involved in the formation of pancreatic intraepithelial neoplasia and promotes the progression of PDAC. However, attempts to target KRAS directly by pharmacological inhibition have been unsuccessful. This has resulted in increased efforts to identify pharmacological targets and nodes associated with the mutated KRAS. The present review discusses the recent progress and prospects of KRAS signaling in pancreatic cancer.

Contents

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an incurable disease that results in mortality. The number of newly diagnosed cases almost equals the annual number of deaths despite advances in surgery and chemoradiotherapy in the past decades. Although the 5-year survival of pancreatic cancer patients has almost doubled over the past decade, it remains low at ~7-8% according to the US National Cancer Institute (1). A lack of early diagnostic strategies, high resistance to chemoradiotherapy and early local or distant metastatic recurrence following surgery are the three predominant factors that contribute to poor outcomes. Further understanding of the biological and molecular mechanisms underlying the initiation and development of PDAC are required. Genetically, cancer progresses as a result of the combined activation of oncogenes and inactivation of tumor suppressors. Similarly, numerous molecular alterations are also required for pancreatic intraepithelial neoplasias (PanIN) lesions to develop into PDAC. Previous studies have established that PDAC is characterized by four signature mutations including mutations in GTPase KRas (KRAS) oncogene and in cyclin-dependent kinase inhibitor 2A (CDKN2A), tumor protein p53 (TP53), and SMAD family member 4 (SMAD4) tumor suppressor genes (2,3). Approximately 90% of pancreatic neoplasms express mutant KRAS, which has been hypothesized to be the initiator of PDAC. However, the development of therapeutic agents targeting KRAS in PDAC remains unsuccessful. The present review discusses recent research regarding KRAS and explores potential therapeutic targets.

2. Initiation

PDAC develops with progressive cellular, morphological and architectural changes from normal ductal epithelium to preneoplastic lesions, and then PanINs and PDAC. The majority of PDAC and early PanIN lesions involve mutations in the KRAS oncogene. Almoguera *et al* (4) and Smit *et al* (5) first established the association between the mutant KRAS gene and PDAC in 1988. To investigate the role of the KRAS oncogene in the onset of PDAC, multiple genetically engineered mouse (GEM) models were established. The first model was the endogenous KRAS-based model, Ptf1a-Cre (6), followed

by the Pdx1-Cre model (7). Pdx1-Cre;LSL-KRAS^{G12D} and Ptf1a-Cre;LSL-KRAS^{G12D} mice were also generated, these are generally referred to as KC mice (8) and express oncogenic KRAS from the earliest stage of pancreatic development. KC mice demonstrate that mutant KRAS is sufficient for the initiation of PDAC. The use of KC mice is a useful tool for pancreatic cancer research, as other signaling pathways and genetic events resulting in pancreatic carcinogenesis may be investigated (9). As tumor suppressor genes are usually lost or inactivated in human PDAC, KC mice have been crossed with mice with non-functional or mutant alleles of CDKN2A or p53 (10,11). The latter model, usually referred to as KPC mice, is currently the most promising preclinical model of PDAC. When treated with standard therapeutic strategies for PDAC, KPC mice are observed to react in same way as human patients (12). PanIN with oncogenic KRAS is able to rapidly progress to PDAC when subjected to inflammatory insult (13).

3. Microenvironment

PDAC is a highly aggressive neoplasm that has a marked fibro-inflammatory microenvironment, promoting cancer induction and growth. GEM models have been used to investigate the role of KRAS on the PDAC microenvironment, which contains large quantities of inflammatory stroma. Immune cells infiltrate around the lowest grade preinvasive lesions, but immunosuppressive cells, including macrophages, regulatory T cells and myeloid-derived suppressor cells (MDSC), predominate in the early response and persist through invasive cancer (14). Phenotype changes of the stellate cells occur earlier than noticeable changes in other pancreatic components (15). Thus, even low levels of KRAS activity generate signals that influence the microenvironment.

By contrast to the majority of other solid tumors, pancreatic tumors are considered to be hypovascularized, although blood vessels are present within the tumor microenvironment as stellate cells produce angiogenic factors (16). In addition to the cellular components, the stroma comprises components of the extracellular matrix, including collagen fibers and hyaluronic acid (17,18). Inactivation of KRAS also results in resolution of the chronic inflammation associated with pancreatic cancer. Thus, KRAS is hypothesized to regulate the production of factors that maintain an active stroma. These factors and their activities remain to be further elucidated, however, Sonic Hedgehog, interleukin-6 (IL-6), and prostaglandin E are considered factors, each of which is expressed in a KRAS-dependent manner (19). Sonic hedgehog (SHH) is one of the ligands of the hedgehog signaling pathway, it is expressed by pancreatic tumor cells (20,21) and functions in a paracrine manner (22), activating hedgehog signaling in the stroma and potentially mediating its maintenance (23). The inflammatory cytokine IL-6 is overexpressed in pancreatic tumors and it important in the development of PanINs in mice (24). Prostaglandin E and prostaglandin E receptor 4 exert a direct effect on stellate cells to stimulate the production of stroma (19). All these factors are generated by sustained high-level KRAS activity.

The immune cells that infiltrate the pancreas also appear to be regulated by KRAS. In mouse models of PDAC, PanINs are infiltrated by immune cells, including those that suppress the immune response, including regulatory T cells, MDSC, and mast cells (25). Tumor cells secrete cytokines, such as granulocyte-macrophage colony-stimulating factor (26,27), which further promotes infiltration of MDSC that inhibit anti-tumor immune responses. KRAS inactivation results in an overall reduction in the number of infiltrating immune cells. Thus, the inflammatory environment of pancreatic tumors also appears to be regulated by KRAS in a paracrine manner, forming part of a KRAS-associated positive-feedback loop of inflammation that requires further elucidation in the future.

Chronic inflammation is known to be a risk factor of pancreatic cancer (28). Although the mechanism is not entirely understood, sustained inflammation contributes to a compromised anti-tumor immune response via the infiltration of immunosuppressive regulatory T cells and MDSC (29,30). In addition, these inflammatory stimuli activate stellate cells and fibroblasts, leading to fibrotic remodeling of the pancreatic tissue, which in turn enhances oncogenic KRAS signaling (29). Neoplastic changes occur under pancreatic inflammation when adult murine pancreas express oncogenic KRAS (31). Thus, oncogenic KRAS signaling is enhanced by inflammatory stimuli, and enhances inflammation and desmoplasia in pancreatic neoplasia.

In addition to intracellular factors, the interactions between the tumor cells and their microenvironment are also controlled by KRAS, although the mechanisms require further elucidation. In iKRAS mice, inactivation of oncogenic KRAS at any stage of carcinogenesis results in reduced proliferation and smooth muscle actin expression in the stroma (32). SHH, secreted by tumor cells, is one of the signals mediating the interaction between the tumor cells and the surrounding fibroblasts within the stroma (20,21), and it also activates paracrine signaling in fibroblasts (22). However, it is probable that additional signals are involved in the regulation of the interactions between KRAS-expressing epithelial cells and the surrounding microenvironment. Oncogenic KRAS mutations and the immune microenvironment may act synergistically to promote the development and progression of PDAC.

4. Metabolic reprogramming

In 1956, Warburg (33) recognized that altered metabolism is a characteristic of cancer. By contrast to normal cells, tumor cells metabolize ~10 times more glucose than lactate, a phenomenon now referred to as aerobic glycolysis or the Warburg effect.

KRAS is key in metabolic reprogramming, particularly in the glycolytic switch (34-37). Oncogenic KRAS was recently demonstrated to regulate metabolic changes in pancreatic cancer cells by increasing the expression of glycolytic enzymes, including hexokinase 1 and 2, glucose transporter 1, phosphofructokinase l and lactate dehydrogenase A (38). KRAS also supports biomass synthesis, of proteins and nucleic acids, and fatty acid synthesis required for pancreatic cancer cell proliferation via stimulation of glucose uptake and channeling of glucose intermediates into the hexosamine biosynthesis and pentose phosphate pathway (38).

Transcriptional reprogramming of key metabolic enzymes (for example glutamate dehydrogenase-1 and glutamic-oxaloacetic transaminase 1) in the glutamine

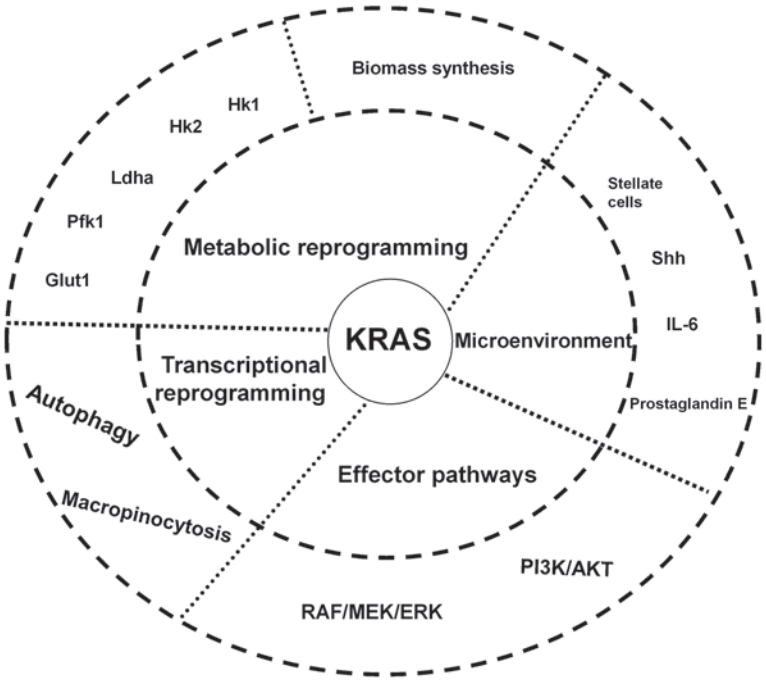

Figure 1. Large number of signaling pathways involving oncogenic KRAS is critical in pancreatic cancer cell proliferation, apoptosis, invasion and metastasis. KRAS, GTPase KRas; Shh, sonic hedgehog; Hk1, hexokinase 1; Hk2, hexokinase 2; Glut1, glucose transporter 1; Pfk1, phosphofructokinase 1; Ldha, lactate dehydrogenase A; IL-6, interleukin 6; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase.

pathway, which is involved in the utilization of autophagy in PDAC, is driven by KRAS (39,40). Inhibition of autophagy in mouse models blocked KRAS tumorigenicity in a wild-type TP53 background, but resulted in PanIN transformation into invasive PDAC in the presence of an oncogenic KRAS mutation and a TP53 deletion (41). KRAS has an additional role in absorbing and degrading the extracellular components of cancer cells, referred to as macropinocytosis. Upregulation of macropinocytosis by KRAS contributes to the metabolic requirements of PDAC cell lines, however, inhibition of macropinocytosis results in slowing of KRAS-transformed cell growth (42,43). Thus, it may be possible to design therapeutic agents to target KRAS, or its effectors, that alter pancreatic cancer metabolism and impair the ability of the cancer cells to maintain high levels of glycolysis (44).

5. Mouse model

Cancer-associated mortality is predominantly due to a lack of early diagnosis and effective therapeutic strategies. However, the underlying molecular mechanisms of PDAC development and progression are little known, thus, the development of mouse models is required, particularly GEM models, to investigate the mechanisms of pancreatic tumorigenesis and reproduce the pathogenesis of PDAC to aid development of diagnostic and therapeutic strategies.

Prenatal mouse models. The expression of a resident $KRAS^{G12D}$ oncogene during early embryonic development was used in the first mouse model that demonstrated natural pathogenesis of human PDAC in mice (7). Briefly, a resident $KRAS^{G12D}$ mouse strain was crossed with transgenic strains that expressed the bacterial Cre recombinase under control of Pdx1 (Pdx1-Cre) or Ptf1a (Ptf1a-Cre), silencing was conducted using a floxed STOP transcriptional cassette (LSL-$KRAS^{G12D}$) to produce Pdx1-Cre;LSL-$KRAS^{G12D}$ and Ptf1a-Cre;LSL-$KRAS^{G12D}$, which express the $KRAS^{G12D}$ oncogene in all pancreatic lineages. Thus, a full series of PanIN lesions that are histologically the same with those in human patients were developed. Mucins, cytokeratin-19, and components of signaling pathways including cyclooxygenase-2, epidermal growth factor receptor, matrix metalloproteinase-7 and transcription factor Hes1 are also expressed in these mouse PanINs (45). There is a long latent period between PanIN lesions and PDAC in a certain percentage of mice. However, induction of mutations observed in human PDAC, including CDKN2A, TP53, liver kinase B1 (LKB1) or SMAD4 resulted in accelerated progression from PanIN lesions to invasive PDAC, a number of the mice also develop metastatic tumors. In recent reviews, the majority of the important GEM PDAC models have been summarized (46-49).

Notch signaling pathways are important in the progression of pancreatic cancer, which has been investigated in GEM models. Deletion of Notch-1 resulted in an increased tumor incidence and progression in Pdx-1-Cre;LSL-$KRAS^{G12D}$ mice, indicating that Notch-1 may be a tumor suppressor gene in pancreatic cancer development (50). However, another previous study demonstrated that deficiency of Notch-2, but not Notch-1, blocked PanIN progression and prolonged survival (51). These findings suggest further investigation into the exact physiological role of Notch-1 in pancreatic cancer initiation and progression is required.

Postnatal mouse models. Though numerous similarities are observed between the PanIN lesions and PDAC in GEM models and those of human patients, the etiology is distinct.

PDAC is not a pediatric disease, which indicates PDAC tumors are more likely to arise as a result of sporadic mutations in adult individuals. In addition, KRAS mutations are not exhibited by the entire pancreas but in certain PDAC cell types. To begin to address these issues, a second generation of GEM models was generated by crossing mice with a resident KRASLSLG12Vgeo allele with double transgenic mice (Elas-tTA;Tet-O-Cre). During late embryonic development, these composite mice express the knocked-in KRASG12 V oncogene in ~20-30% of acinar cells (31). Notably, the latent period and penetrance of PanIN lesion development are similar to those expressing the $KRAS^{G12D}$ oncogene. The above model enables expression of KRAS oncogene to be activated in a controlled temporal manner by feeding the mice with doxycycline (52).

In PDAC, serine/threonine-protein kinase B-raf (BRAF) or phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (PIK3CA) activation is uncommon (53). However, GEM models with BRAF or PIK3CA mutations may provide key information to aid understanding of PDAC development. For example, the expression of the BRAF V600E mutation in early pancreatic precursors results in embryonic lethality. However, in P14 mice that express the same BRAF V600E mutation, activation of the Pdx1-CreERT2 (estrogen receptor 2) transgene by exposure to tamoxifen results in widespread PanIN development (54). Notably, these PanINs did not progress to PDAC tumors within one year. However, with the same Pdx1-CreERT2 transgene, activation of the PIK3CA H1047R oncogene did not induce any PanIN lesions. This suggests that it is the RAF/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway activated when KRAS oncogenes initiate PanIN lesions rather than the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT signaling pathway. This indicates the importance of the RAF/MEK/ERK signaling pathway in tumor initiation and maintenance. Furthermore, future therapeutic strategies may be developed to target it.

Hereditary mouse models. Among patients with pancreatic cancer, ~10% have a family history of the disease. There is a 2 times greater risk of pancreatic cancer when a first-degree relative is diagnosed. Families carrying germline mutations in certain genes, including breast cancer 2, early onset (BRCA2), CDKN2A, LKB1, protease serine 1 and partner and localizer of BRCA2 also have an increased risk (55). Through mixing the KC strain of mice with mice with a truncated BRCA2 gene, two GEM models for hereditary pancreatic cancer have been produced. PDAC formed in these mice with high penetrance and a shorter latency period compared with those carrying wild-type BRCA2 alleles (56). However, in a similar study, a $KRAS^{G12D}$-background mouse with BRCA2 homozygously inactivated developed acinar carcinoma, not PDAC (57). The KC mouse model with a conditional floxed LKB1 allele also resulted in an increased number of PanINs, and complete penetrance and shorter latency of PDAC tumor formation (58). Notably, where the KRAS oncogene is not observed, homozygous loss of BRCA2 or LKB1 has different results in early pancreatic precursors. Pdx1-Cre;LKB1lox/lox mice have very short latency periods prior to development of pancreatic mucinous cystadenomas (58), though knockout of BRCA2 does not induce histological alterations (57). These observations suggest that there are a variety of methods to control the malignant transformation of pancreatic cells.

Other mouse models. In 1985, Hanahan (59) described the widely used RIP-Tag model, which is an important GEM model of pancreatic endocrine tumor. Models of intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) were also later developed. For example, in the pancreas of KC mice, Smad4 deletion was observed to induce the development of IPMNs and MCNs (60,61). These cystic preneoplasms, if not resected, have the potential to progress to invasive PDAC. In 2007, Siveke *et al* (62) indicated that co-expression of transforming growth factor (TGF)-α and $KRAS^{G12D}$ results in the development of cystic papillary neoplasms similar to IPMNs. Furthermore, deletion of the transcriptional intermediary factor-1 γ, which is thought to be involved in TGF-β signaling in pancreatic progenitor cells, with $KRAS^{G12D}$ induced IPMNs (63). These GEM models illustrate that the PanINs and cystic neoplasms that progress to PDAC are associated with alterations in the TGF-α and TGF-β signaling pathways.

6. Inhibitors

The KRAS gene has been demonstrated to be important in the development of PDAC, demonstrating the development of KRAS inhibitors is required. Blocking the KRAS GTP binding site directly prevents KRAS signaling. However, effective therapies that directly target mutated KRAS remain unavailable, thus, research has focused on targeting KRAS indirectly. Farnesylated KRAS following translation is translocated to the membrane and the Ras-activating proteins located there. It is then activated by guanine nucleotide exchange factors (Ras-GEFs). Farnesyltransferase inhibitors (FTIs) are important in the post-translational modification of KRAS activation. Certain FTIs, including lonafarnib and tipifarnib, have been tested clinically tested, though the results are not yet satisfactory in treating KRAS-driven tumors (64). This failure may be due to the three different types of Ras proteins.

The majority of successful results of FTIs in preclinical studies have focused on GTPase HRas (HRAS)-dependent tumors (65). Compared with HRAS, KRAS may be geranylgeranylated by inhibiting farnesyltransferase (66). Via the alternate post-translational modification of farnesylation, KRAS may be localized to the membrane and so be activated. This has led to the development of potential therapeutic strategies to prevent KRAS reaching the membrane. Deltarasin is an inhibitor that binds to the farnesyl-binding pocket of phosphodiesterase (PDE) (67). Following farnesylation, KRAS interacts with PDE and is translocated to the membrane (68). Salirasib is another inhibitor that limit KRAS activity in the membrane. Unlike PDE, Salirasib removes the farnesylated protein from the membrane, thus blocking KRAS activity (69). Salirasib has shown potential as a KRAS inhibitor in preclinical and clinical trials against PDAC (70).

When KRAS cannot be blocked from reaching the membrane, other therapeutic strategies are required to prevent activation of KRAS on the membrane. Patgiri *et al* (71)

designed a small-molecule α-helix mimic, using the hydrogen bond surrogate, to block the exchange of GDP for GTP, and thus inhibit the interaction between KRAS and its Ras-GEF SOS. Post-translational acetylation of KRAS alters the ability of SOS to exchange GDP for GTP, however, further research is required to elucidate the role acetylation has in the activity of mutant KRAS.

The RAF/MEK/ERK and PI3K/AKT signaling pathways are the targets of an increasing amount of research and numerous inhibitors targeting these signaling pathways are already being tested in clinical trials. In $KRAS^{G12D}$-driven GEM models, inhibition of PI3K signaling has been demonstrated to be efficient at inhibiting growth *in vivo* (72). Inhibition of MEK1/2 has demonstrated suppression of cell growth in cell lines of orthotopically transplanted human and mouse PDAC. Preclinical studies of non-small cell lung cancer (NSCLC) have also demonstrated successful results for this potential therapy (73).

Dual-pathway inhibition has demonstrated promising results, however, its toxicity is markedly higher than single-agent therapy (74). To ameliorate this, tissue-specific effectors required for the activation of the two signaling pathways should be targeted. In preclinical studies of NSCLC, Molina-Arcas *et al* (75) demonstrated dual-pathway inhibition via inhibiting IGF1R and MEK. However, further investigation is required to determine the efficacy of dual signaling pathway inhibition against KRAS-driven PDAC.

7. Summary

Although oncogenic KRAS has been associated with PDAC for over 20 years, pharmacological attempts to target KRAS directly have been unsuccessful. Single downstream effector inhibition may only be modestly effective as oncogenic KRAS activates multiple downstream signaling pathways (Fig. 1). More studies are required to further elucidate the underlying mechanisms of PDAC initiation and progression. Comprehensive investigation into PDAC may provide potential therapeutic strategies against pancreatic cancer for the future.

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