Trends in Cancer

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Review

Next batter up! Targeting cancers with KRAS-G12D mutations

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KRAS is the most frequently mutated oncogene in cancer. Activating mutations in codon 12, especially G12D, have the highest prevalence across a range of carcinomas and adenocarcinomas. With inhibitors to KRAS-G12D now entering clinical trials, understanding the biology of KRAS-G12D cancers, and identifying biomarkers that predict therapeutic response is crucial. In this Review, we discuss the genomics and biology of KRAS-G12D adenocarcinomas, including histological features, transcriptional landscape, the immune microenvironment, and how these factors influence response to therapy. Moreover, we explore potential therapeutic strategies using novel G12D inhibitors, leveraging knowledge gained from clinical trials using G12C inhibitors.

Highlights

G12D mutation is the most common KRAS mutation detected in carcinomas.

G12D mutation confers a unique structural conformation that influences downstream signaling and may lead to its potent oncogenic activity.

Invasive mucinous adenocarcinoma of the lung displays histological features of gastrointestinal cancers and a predominance of KRAS-G12D mutations.

Adenocarcinomas of the lung, pancreas and colon driven by KRAS-G12D mutation display an immunosuppressive tumor microenvironment.

Mutations in tumor suppressor genes found to co-occur with KRAS-G12D influence tumor biology and response to therapy.

KRAS-G12D inhibitors may need to be tailored according to tissue of origin and considered in the context of the co-mutational genomic landscape.

G12D is the most common KRAS-activating mutation

KRAS is a **GTPase** (see Glossary) that cycles between a GDP-bound inactive and GTP-bound active state, playing an important signal transduction role in the regulation of cell proliferation and survival. KRAS mutations commonly occur in carcinomas and **adenocarcinomas** (Figure 1A), with activating mutations most commonly occurring as single nucleotide substitutions in four hotspot codons – 12, 13, 61, and 146 [1]. Codon 12 is the most frequently mutated of all four hotspot codons, with the G12D mutation generally the most prevalent, followed by G12V, G12C, and others (Figure 1B). Depending on the cancer type, the G12D mutation accounts for 20–50% of KRAS-mutated cancers, including, 50% of ampullary carcinoma, 48% of appendiceal adenocarcinoma, and 44% of cholangiocarcinoma. Low-grade serous or endometrioid ovarian cancer and lung adenocarcinomas are among the few cancers in which G12D mutation is not the most frequent KRAS mutation. In lung adenocarcinomas, G12C (c.34G>T) mutations are more frequent than G12D (c.35G>A), likely due to prevalence of mutational signature 4 (associated with tobacco smoking), characterized by C>A/G>T substitutions [2]. In contrast, KRAS-G12D (c.35G>A) mutations are more likely attributable to clock-like mutational signatures 1 and 5, characterized by increased C>T/G>A substitutions [1]. In pancreatic adenocarcinoma, the KRAS-G12D mutation is associated with worse overall survival compared with wild-type KRAS, KRAS-G12R, or KRAS-G12V mutations [3,4]. In colorectal cancer (CRC) there is a clear survival advantage in KRAS wild-type compared with KRAS mutant in response to anti-**epidermal growth factor receptor (EGFR)** therapies [5]; however, inconsistent data pertaining to the behavior of distinct KRAS variants exist. Similarly, the prognostic impact of distinct KRAS variants in lung adenocarcinoma remains unclear, with some evidence suggesting that G12D mutations serve as a predictor of poor survival [6,7].

The emergence of allele-specific KRAS inhibitors presents novel opportunities for targeting G12D cancers. However, it also sparks questions regarding the response of these tumors across different tissues of origin and the feasibility of predicting responsive tumors or identifying effective combination therapies in advance. This Review aims to explore the distinctive biochemical and biological characteristics of tumors carrying G12D mutations. The potential impact of these

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attributes on the therapeutic response to KRAS-G12D inhibitors will be examined with the goal of informing clinical trials with optimized designs, ultimately leading to improved outcomes for patients.

Biochemical and structural properties of KRAS-G12D

In a normal cellular state, KRAS bound to GTP activates the **RAF/MEK/ERK**, **PI3K/AKT/mTOR** and **RALGDS/RAL** pathways to drive cellular proliferation and promote survival. The core catalytic machinery of KRAS is in the G-domain, which consists of six β-sheets and five α-helices, with the active site containing a phosphate binding loop (P-loop) and two switch regions, I and II (Figure 2A). The active site interacts closely with the phosphate groups of GTP and mediates GTPase activity. The switch regions have different conformations when bound to GDP or GTP and are responsible for binding effector and regulator proteins. For example, when bound to GTP, switch I has two conformation states: state 1 is open away from the nucleotide, and state 2 is closed over the nucleotide and can interact with the three RAS effector proteins RAF, phosphatidylinositol 3-kinase (PI3K), and Ral guanine nucleotide dissociation stimulator (RALGDS) [8,9].

KRAS-activating mutations primarily occur within the active site near the γ-phosphate of GTP at residues G12 and G13 within the P-loop or the catalytic residue Q61. These mutations lead to increased levels of KRAS–GTP; most commonly through impairment of intrinsic and GTPase-activating protein (GAP)-catalyzed **GTP hydrolysis** [10,11], or in the case of G13D and A146T mutants, through increased intrinsic and guanine nucleotide exchange factor (GEF)-catalyzed nucleotide exchange activity [11,12]. G12D has been shown to have an intermediate intrinsic and GAP-mediated GTP hydrolysis rate compared with other G12 and G13 mutants, with mutations such as G12A significantly reducing intrinsic hydrolysis, and G12C exhibiting wild-type levels [11]. During intrinsic GTP hydrolysis, a bridging water molecule is present within H-bonding distance of Y32, the γ-phosphate of GTP, and Q61, and is proposed to neutralize the negative charge that develops during hydrolysis (Figure 2A). In all G12 and Q61 mutants, except for G12D, the bridging water molecule is lost and Y32 creates a direct H-bond with the γ-phosphate severing the connection between switch I and nucleotide-sensing residues [10]. In contrast, G12D is unique in that the aspartate residue in the mutant protein leads to the projection of a bulkier side chain that is negatively charged [8]. This causes the bridging water molecule to either be replaced with a side-chain oxygen atom, or it be present and G12D replacing Q61 in its interaction with the water molecule (Figure 2A). As a result, the charge distribution during hydrolysis is perturbed. In addition, as the bulky sidechain links the two switch regions in the active site together, there is a stabilization of the state 2 conformation that binds to effector proteins and impairs the binding of regulatory GAP proteins. Importantly, the presence of the bulky aspartate maintains the connection between switch I and nucleotide sensing residues unlike in all other mutants [8], which may contribute to its high prevalence and oncogenic potential in adenocarcinomas (Figure 1B).

The structural conformation of G12D influences its downstream signaling. The G12D mutation has been shown to have the greatest binding affinity for PI3K and low affinity for RAF [13,14]. In pancreatic and lung adenocarcinoma *in vitro* models, G12D mutants were shown to exhibit elevated PI3K/AKT signaling compared with other KRAS alleles [14,15], with G12D lung adenocarcinomas being significantly less sensitive than G12C to mitogen-activated extracellular signal-regulated kinase (ERK) kinase (MEK) inhibition [16,17]. In contrast, G12D mouse colon samples displayed increased phosphorylated ERK levels compared with wild-type or A146T mutants [12]. G12D mutant mouse colon organoid models suppressed phosphorylated AKT expression compared with wild-type or A146T mutants, and responded to ERK but not AKT inhibitors [18]. These data suggest that biological pressures from within tissues can also alter downstream KRAS-G12D signaling. KRAS-G12D mutations have been shown to elicit distinct gene and protein expression profiles compared with other KRAS mutations in a tissue-specific manner

Glossary

Adenocarcinoma: carcinomas are cancers that start in the epithelial tissue of your skin or internal organs. Adenocarcinoma is a subtype of carcinoma that grows in the glands that line the insides of organs.
Adenoma: a type of noncancerous or benign tumor of epithelial tissue.
Anti-PD-1: antibody targeting PD-1 that blocks the activity of the PD-1 and PD-L1 immune checkpoint to overcome the OFF signal delivered to T cells.
Autochthonous: autochthonous mouse models for human cancers are obtained by initiating tumors in a normal cell within the intact organism.
Cellular senescence: process in which cells cease dividing and undergo distinctive phenotypic alterations.
Epidermal growth factor receptor (EGFR): receptor for members of the epidermal growth factor family of extracellular protein ligands. It is involved in pathways that regulate cellular proliferation and survival.
Genomic landscape: genes that are commonly mutated within a cancer type.
GTPase: enzyme that catalyzes the hydrolysis of GTP to GDP and inorganic phosphate.
GTP hydrolysis: hydrolysis of the third (γ) phosphate of GTP to create GDP and inorganic phosphate.
Isogenic: cell lines that are genetically matched.
Mucin: large, heavily glycosylated protein that combined with water forms a mucus layer that serves as a physical barrier to protect epithelial cells from pathogens and mechanical damage.
Oncogene: mutated gene that has the potential to cause cancer.
Oncogenic potential: potential of a gene mutation to cause cancer.
PI3K/AKT/mTOR pathway: intracellular pathway that regulates cell growth, motility, survival, metabolism, and angiogenesis.
Programmed cell death ligand 1 (PD-L1): immune checkpoint molecule expressed by tumor cells that binds to PD-1 receptor on T cells and suppresses their activity.
Proteomic analysis: analysis of the structure and function of all the proteins within a cell or other biological context.
RAF/MEK/ERK pathway: critical pathway that regulates cellular proliferation, differentiation, and survival.
RALGDS/RAL pathway: intracellular pathway that regulates gene

[13,17,19]. In lung adenocarcinoma patients harboring G12D mutations, tumor cells showed downregulated expression of chromosome maintenance, DNA double-strand break repair, and **cellular senescence** pathways compared with wild-type, pathways that were unaffected in tumors bearing other KRAS mutations [17]. Across tumor types, **proteomic analysis** of colon and pancreas of genetically engineered Kras-G12D mice showed similar but also tissue-specific pathway expression [19]. These potential tissue specific effects may influence what therapies are evaluated in combination with KRAS-G12D inhibitors.

Strong evidence distinguishing G12D from other KRAS mutations is observed in KRAS-mutant cancer models. G12D alone or in combination with tumor suppressor mutations consistently displays the highest **oncogenic potential** compared with other KRAS mutations in pancreatic, lung and colorectal cancer *in vitro* and *in vivo* models [12,15,17,18,20,21]. In one study [21], mice were infected with a barcoded library of Kras mutations concurrently with loss of commonly mutated tumor suppressors *p53* or *Lkb1* (*STK11*) to initiate adenocarcinomas. G12D mutations were found to be the primary driver in the development of spontaneous pancreatic and lung adenocarcinomas in mice. Counterintuitively, G12C, the most common mutation in lung adenocarcinoma (Figure 1B), exhibited only partial oncogenic potential, suggesting that tumor-extrinsic factors such as tobacco smoke may augment the ability of G12C to drive malignant transformation [7,22]. This notion, however, remains to be investigated. Similar results were seen in **isogenic** mouse embryonic fibroblasts cell lines harboring G12, G13 and Q61 mutations, where only G12D mutant cells formed tumors in the lungs of recipient mice [17].

All these studies suggest that the high prevalence and oncogenic potential of the G12D mutation may be due to its favorable structural conformation and downstream signaling. Whether other factors beyond the biochemical properties and structure of G12D contribute to its high oncogenic potential remains unclear. The structure of G12D compared with other KRAS mutations has also affected the design of allele-specific KRAS inhibitors (Figure 2B) and is discussed below (see KRAS-G12D inhibitors).

Features of KRAS-G12D adenocarcinomas

Histopathology

KRAS-G12D cancers display varying histopathological features across multiple cancer types. However, within lung adenocarcinomas, there is an interesting association of different types of KRAS mutation with distinct histological features. While KRAS-G12C mutations are the most common KRAS mutation in nonmucinous lung adenocarcinoma, invasive mucinous adenocarcinoma (IMA) of the lung (Box 1) [23] shows a predominance of KRAS-G12D and G12V mutations, and exhibits histological and immunohistochemical features of gastrointestinal differentiation. IMAs of the lung account for 3–10% of lung adenocarcinomas and are composed of goblet and/or columnar cells with abundant cytoplasmic **mucin** [23]. The prevalence of recurrent mutations in *KRAS*, *ERBB2*, and *SMAD4* seen in IMAs of the lung is akin to the genomic landscape of pancreatic and colorectal adenocarcinomas (Figure 2A) [23–25]. These observations suggest that KRAS-G12D and KRAS-G12V may contribute to malignant transformation via a conserved mechanism, giving rise to phenotypically similar tumors in different tissue contexts.

Genomic landscape

While mutations in *KRAS* occur in premalignant lesions, concurrent genomic alterations are often required for sustained tumor growth. Indeed, genomic alterations associated with a KRAS-G12D mutation vary across cancer types (Figure 3B), with the **genomic landscape** more consistent with the tissue of origin and less influenced by the allele-specific KRAS mutation (Figure 3C). For example, in colorectal adenocarcinoma, G12D mutations are frequently observed

transcription, cell proliferation, cell survival, and actin organization.
Syngeneic transplantation: syngeneic models are transplantation models obtained by injecting a recipient of a specific genetic background with cell lines previously established through isolation of tumor cells from a mouse of the same genetic background.
Tumor heterogeneity: describes the differences between tumors of the same type in different patients (intertumoral heterogeneity), the differences between cancer cells within a single tumor (intratumoral heterogeneity), or the differences between a primary (original) and a secondary tumor.
Tumor mutational burden: total number of mutations found in the DNA of cancer cells.
Tumor suppressor gene: type of gene that regulates cell growth and when mutated (typically loss-of-function mutations) uncontrolled cell growth may occur and lead to cancer development.
Xenograft: transplantation of an organ, tissue, or cells to an individual of another species.

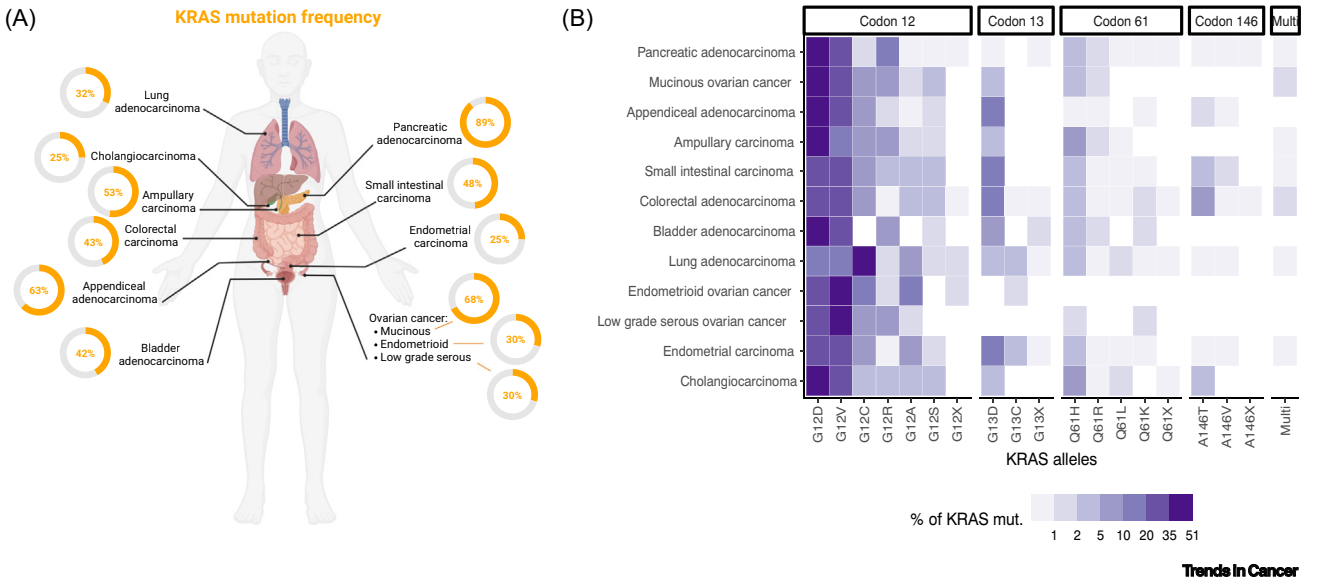
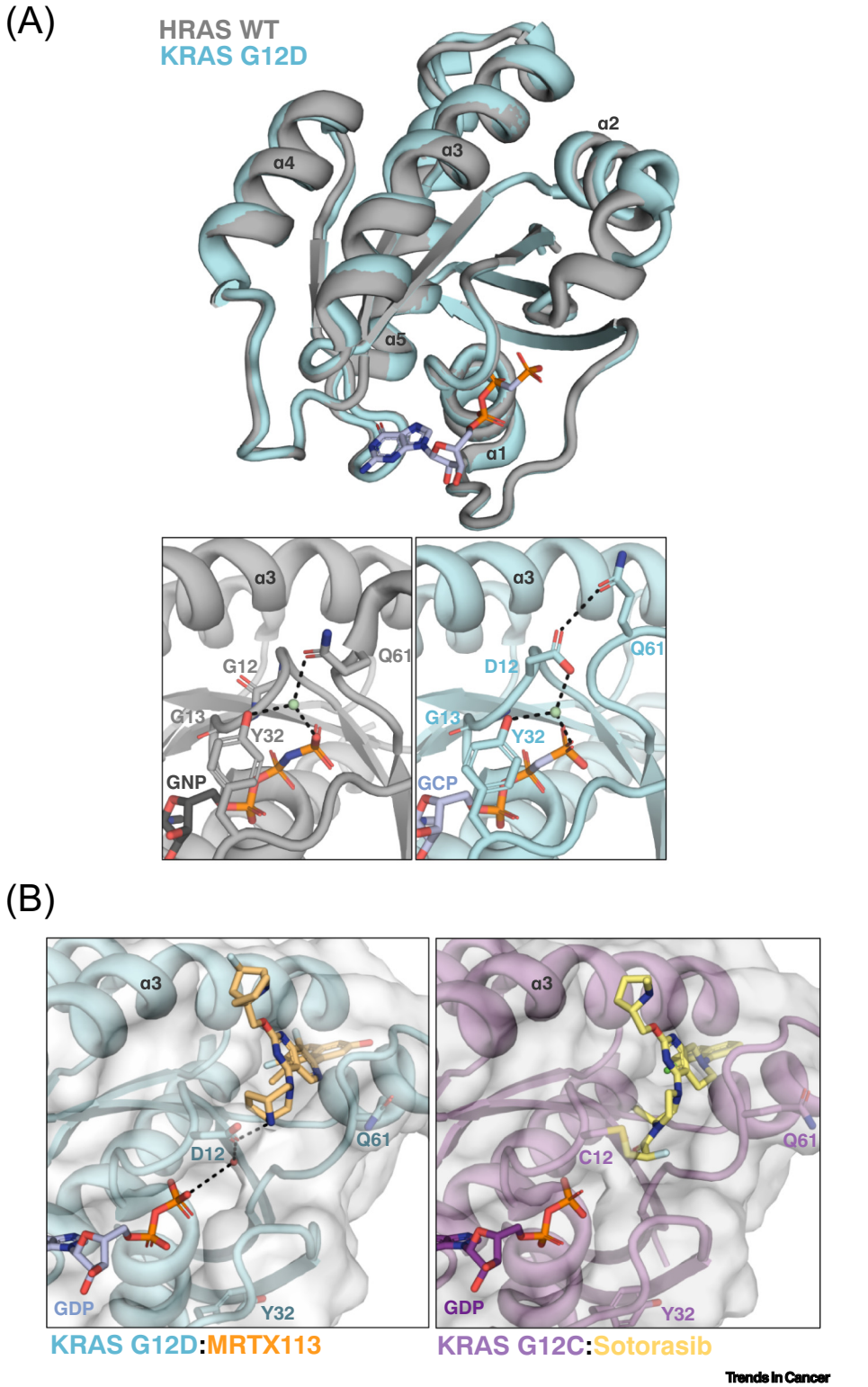


Figure 1. KRAS mutation frequency in adenocarcinomas from the Genie Cohort v13.0. (A) Adenocarcinomas with frequent KRAS somatic mutations (≥20% alteration frequency and ≥20 alteration count). (B) Distribution of KRAS alleles across hotspot codons 12, 13, 61, and 146. Multiple hotspot (multi) includes patients with mutations in more than one hotspot codon. Color of tiles reflects the percentage of KRAS mutations that are the indicated KRAS allele in each adenocarcinoma. Patients with conflicting inter-sample mutations or diagnosis with multiple adenocarcinomas were excluded from analysis. Oncotree codes: pancreatic adenocarcinoma (PAAD; *n* = 4718), mucinous ovarian cancer (MOV; *n* = 106), appendiceal adenocarcinoma (APAD, MAAP; *n* = 421), ampullary carcinoma (AMPCA, PAMPCA; *n* = 220), small intestinal carcinoma (DA, SBC, SIC; *n* = 344), colorectal adenocarcinoma (COAD, COADREAD, READ; *n* = 10 707), bladder adenocarcinoma (BLAD; *n* = 139), lung adenocarcinoma (LUAD; *n* = 13 314), endometrioid ovarian cancer (EOV; *n* = 204), low grade serous ovarian cancer (LGSOC; *n* = 252), endometrial carcinoma (UCEC, UEC; *n* = 2678), cholangiocarcinoma (CHOL, EHCH; *n* = 792).

with mutations in **tumor suppressor genes** *APC*, *TP53*, and *SMAD4* and the **oncogene** *PIK3CA* (Figure 3B). Using CRC organoid models, co-mutations in these genes led to growth-factor-independent growth and the formation of tumors when engrafted under the kidney subcapsules of mice, while organoids harboring only KRAS-G12D failed to engraft [26]. In pancreatic adenocarcinoma, KRAS-G12D mutations are observed with mutations in tumor suppressors *TP53*, *CDKN2A* (also referred to as *Ink4a/Arf*), and *SMAD4* (Figure 3B). Activation of Kras-G12D in pancreatic epithelial lineages of the embryonic pancreas resulted in a spectrum of low-grade pancreatic intraepithelial neoplasias (panINs), but additional expression of mutant p53 (R270H) or biallelic loss of $p16^{Ink4a}$/$p19^{Arf}$ is essential for the development of pancreatic ductal adenocarcinoma [27–29]. In lung adenocarcinoma, common concurrent mutations are observed in tumor suppressors *TP53*, *STK11*, and *KEAP1* (Figure 3B). Enforced expression of Kras-G12D in lung epithelial cells was sufficient to induce benign **adenoma** formation [21]; however, concomitant loss of *p53*, *Lkb1* (*STK11*), and/or *Keap1* was required to drive tumor progression [21,30]. Different co-mutations have also been associated with specific KRAS mutations within cancer types [1]. In lung adenocarcinoma *STK11* and *ATM* mutations are significantly enriched in cancers with non-G12D mutations, with lower rates of co-mutation observed for G12D [17,31]. Similarly, G12D is reported to have reduced co-mutations with *TP53* compared with other KRAS alleles in CRC [1].

Concurrent genomic alterations can dictate the phenotype and behavior of KRAS-mutated adenocarcinomas. It is particularly evident in KRAS-mutated lung adenocarcinoma where concurrent mutations in tumor suppressor genes *TP53*, *STK11*, *KEAP1*, and *CDKN2A* are key determinants of **tumor heterogeneity** and response to therapies (Box 2). For example, co-mutations in *KEAP1* are associated with decreased overall and progression-free survival, while *TP53* mutations fail to serve as a prognostic biomarker [32]. Co-mutation of *KEAP1* renders



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Box 1. Lung IMA shows features of intestinal and pancreatobiliary tract cancers

IMA, formerly known as mucinous bronchioloalveolar carcinoma, represents 5% of lung adenocarcinoma cases and is more frequently seen in never-smoker female patients [64,65]. Diagnostically, IMA of the lung can mimic pneumonia, due to its multifocal clinical presentation coupled with multilobar consolidation [23]. IMAs are characterized by distinct histopathological features that are rarely observed in other lung adenocarcinomas. Tumor cells exhibit goblet and/or columnar morphology with abundant intracytoplasmic mucin and basally located nuclei [23]. Consistent with their higher frequency in never-smoker patients, IMAs are associated with a considerably low TMB and lack mutational signature 4, indicative of tobacco smoke exposure. Conversely, mutational signature 1, the result of an endogenous mutational process was most frequently observed in the majority of IMAs of the lung, with signatures associated with APOBEC activity (signatures 2 and 13) and MMR deficiency (signature 6) also observed in a subset of patient samples [64].

KRAS mutations are the most prevalent oncogenic drivers in IMAs, with up to 86% of IMAs driven by KRAS compared with one-third of non-IMAs of the lung [64,65]. There is a preponderance of KRAS-G12D (~36%) and G12V (~32%) variants in IMAs, an enrichment not seen in nonmucinous lung adenocarcinoma in which G12C variants are more frequently observed [65]. While survival data for IMAs have historically been limited, owing to its paucity, a recent study of 200 IMA of the lung revealed an association between KRAS altered IMAs and favorable overall survival [65]. In addition to oncogenic driver mutations in *KRAS*, recurrent mutations in *CDKN2A*, *STK11*, *ERBB2/3*, *SMAD4*, *NKX2-1*, and *NRG1* gene rearrangements have been identified [25]. Of note, loss-of-function mutations in NK2 homeobox 1 (NKX2-1), also known as TTF-1, was seen in ~19% of IMAs [66], suggesting that NKX2-1 plays a crucial role in IMA tumorigenesis. Indeed, concomitant inactivation of *Nkx2-1* in a KrasG12D-driven autochthonous lung cancer model led to the formation of mucinous adenocarcinomas with similarities to IMA seen in humans [67].

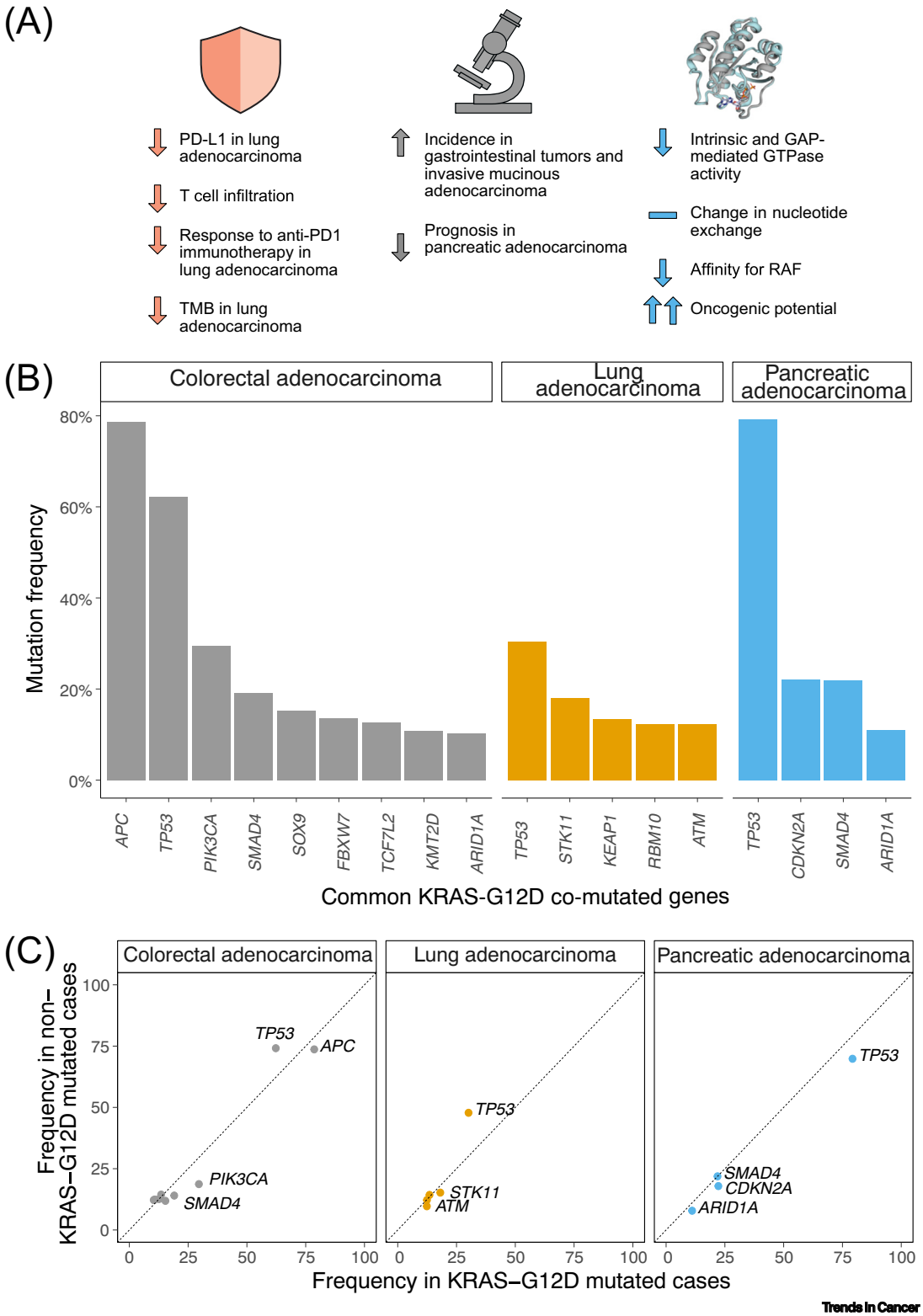
KRAS-G12C tumors more resistant to KRAS-G12C inhibitors adagrasib (Mirati) and sotorasib (Amgen) [33,34], but it remains unclear whether similar effects will be observed with KRAS-G12D inhibitors.

Immune microenvironment

There is notable evidence suggesting that the G12D mutation confers a more immunosuppressive tumor microenvironment and heightened resistance to immunotherapy compared with other KRAS mutations (Figure 3A). In a murine subcutaneous injection model, tumors from isogenic mouse non-small cell lung cancer (NSCLC) cells harboring different G12 mutations were treated with **anti-PD1** monotherapy. Compared to other KRAS mutations, G12D mutant tumors displayed increased resistance to anti-PD1 therapy, reduced expression of **programmed death ligand 1 (PD-L1)** expression on tumor cells and decreased cytotoxic $CD8^+$ T cell infiltration into tumors [35]. A similar observation has been made in NSCLC patients, with KRAS-G12D tumors exhibiting reduced PD-L1 expression and $CD8^+$ T cell infiltration compared with tumors harboring G12C mutations [7]. Similarly, colorectal adenocarcinoma tumors with G12D and G12V mutations had decreased density of tumor infiltrating $CD3^+$ lymphocytes compared with other G12 or G13D mutations in patient samples [36]. In an independent patient cohort, G12D tumors displayed decreased $CD8^+$ T cell infiltration compared with wild-type [37]. Similarly, expression of Kras-G12D decreased infiltration of T cells and increased infiltration of myeloid derived suppressor cells compared with wild-type in mouse models of G12D driven CRC [38].

Factors such as PD-L1 expression on the tumor cells, **tumor mutational burden** (TMB) [39,40], and co-occurring genetic alterations (Box 2) have all been shown to influence response to immunotherapy.

Figure 2. The G12D mutation elicits a unique conformational structure compared with other KRAS mutations. (A) The active site of wild-type HRAS bound to GTP analog GppNHp (GNP) (PDB: 3K8Y) and KRAS-G12D bound to GTP analog GppNHp (GNP) (PDB: 4DSN). HRAS is depicted in grey, KRAS-G12D in light blue. The network from Y32 to the γ-phosphate of the GTP analog and bridging water molecule (green) is shown in black dashed lines. In the KRAS-G12D structure, the bridging water molecule is present and the oxygen atom in the D12 side chain takes the place of the Q61 side chain in making a H-bond to the bridging water molecule. (B) The active site of KRAS-G12D (light blue) bound to GDP with KRAS-G12D inhibitor MRTX1133 (orange) bound to the switch II pocket (PDB: 7RPZ). The piperazinyl group forming an ionic bond with the mutant aspartate is shown in black dashed lines. KRAS-G12C (purple) bound to GDP and KRAS-G12C inhibitor sotorasib (yellow) (PDB: 6UT0). The irreversible covalent bond between the reactive warhead of sotorasib and the mutant cystine is depicted.



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Box 2. *TP53*, *STK11/LKB1*, and *CDKN2A* co-mutations define subsets of KRAS-mutant lung adenocarcinoma

Recent studies have uncovered the heterogeneous nature of KRAS-mutant lung adenocarcinomas [68]. Seminal work by Skoulidis *et al.* identified three distinct transcriptional subgroups of KRAS-mutant lung adenocarcinoma that correlated with genetic alterations in key tumor suppressor genes: *TP53* and *STK11* (also known as *Lkb1*), which can be further enriched by somatic alterations in *KEAP1*, and biallelic inactivation in *CDKN2A/CDKN2B* [69]. Apart from an enrichment in KRAS-G12D mutations in the *CDKN2A/CDKN2B* in some cohorts, there was no clear association between the three transcriptional subtypes and distinct KRAS variants, suggesting that the co-mutations are key determinants of the heterogenous phenotype of KRAS-mutant lung adenocarcinoma. Critically, emerging evidence indicates that these co-occurring mutations can directly influence the tumor phenotype, behavior, and therapeutic response. Co-mutations in *KEAP1* are associated with decreased overall survival (OS) and progression-free survival (PFS) in KRAS-mutant lung adenocarcinoma patients, while *TP53* mutations fail to serve as a prognostic biomarker [32]. Response rates to chemotherapy and immune checkpoint inhibitors are similarly dictated by the presence of concurrent *KEAP1* or *STK11* mutations. In the context of immune checkpoint inhibitor efficacy, *STK11* and *KEAP1* mutation status was associated with significantly worse PFS and OS to PD-L1 inhibition, a response not observed in KRAS wild-type patients [70]. Studies in Kras-G12D syngeneic and autochthonous preclinical models suggest that this is likely, in part, due to low PD-L1 expression and an immune cold tumor immune microenvironment [71,72]. Immense interest is now focused on identifying ways to enhance immunotherapeutic responses in these aggressive genetic subtypes of KRAS-mutant lung adenocarcinoma.

In lung adenocarcinoma, G12D mutations are associated with lower TMB, likely due to its high prevalence in never-smokers [7,22], and reduced response to anti-PD-1 monotherapy compared with other KRAS mutations [7]. However, this survival disadvantage is lost when patients are treated with chemoimmunotherapy, suggesting that combinatorial approaches may be advantageous for G12D patients [7]. The KRAS-G12C inhibitor adagrasib in combination with anti-PD1 has also shown synergistic effects for the treatment of NSCLC (KRYSTAL-7 and KRYSTAL-1 trials). Considering the reduced effectiveness of anti-PD-1 therapy in KRAS-G12D patients, it will be intriguing to explore whether combining a KRAS-G12D inhibitor with immunotherapy yields comparable efficacy to adagrasib. In pancreatic, colorectal and endometrial cancers there are little reported differences in TMB between KRAS mutations [41]. Immunotherapy and chemoimmunotherapy have yielded limited success in the treatment of pancreatic adenocarcinoma [42], which has a high prevalence of G12D mutations and typically exhibits low TMB. The presence of highly immunosuppressive fibrotic stroma [43] may contribute to the limited response to immunotherapy. In preclinical models of pancreatic adenocarcinoma, treatment with KRAS-G12D inhibitor MRTX1133 resulted in alterations to the fibrotic stroma, including increasing collagen deposition and a higher abundance of myofibroblastic cancer-associated fibroblasts [44]. These findings suggest that investigating the combination of KRAS-G12D inhibition with immunotherapy is warranted in the context of pancreatic adenocarcinoma.

Metabolism

Oncogenic mutations in KRAS reprogram a myriad of metabolic processes in cancer cells that promote tumor cell growth and survival [45,46]. While emerging evidence supports the notion that specific KRAS mutations can confer unique biological activities, our understanding of whether distinct KRAS mutations are associated with different metabolic phenotypes remains limited. The metabolic profile of KRAS variants was evaluated in isogenic SW48 CRC cells engineered to express wild-type KRAS, codon 12, 13, 61, or 146 KRAS variants [47]. While the metabolic profiles of SW48 cancer cells harboring codon 12 or 13 KRAS mutations were

Figure 3. Biological features and genomic landscape of KRAS-G12D adenocarcinomas. (A) Immune evasion, histological, clinical, and biochemical features of KRAS-G12D adenocarcinomas that are common across cancer types. (B) Frequency of top KRAS-G12D co-mutated cancer genes in the Genie Cohort v13.0 in colorectal (n = 1280), lung (n = 511), and pancreatic (n = 1678) adenocarcinoma (≥10% frequency and ≥100 profiled cases). (C) Frequency of co-mutated cancer genes in the Genie Cohort v13.0 in KRAS-G12D mutated cases compared to non-KRAS-G12D cases (colorectal n = 9990, lung n = 10 801, and pancreatic n = 4499). Only OncoKB annotated cancer genes were included. Patients with conflicting inter-sample mutations or diagnosis with multiple adenocarcinomas were excluded. Oncotree codes: colorectal adenocarcinoma (COAD, COADREAD, READ), lung adenocarcinoma (LUAD), pancreatic adenocarcinoma (PAAD).

generally distinct to that seen in cells expressing wild-type KRAS, few changes detected were unique to G12D lines. One exception is the consumption of extracellular methionine, with G12D cell lines displaying similar consumption levels to wild-type control lines [47], suggesting that methionine is not a metabolic vulnerability for KRAS-G12D CRC. Isogenic studies in the NSCLC cell line NCI-H1299 [48] yielded similar findings to that seen in SW48 lines. However, it is important to emphasize that these studies have primarily been conducted *in vitro*, and the extent to which these observed phenotypes are recapitulated *in vivo* remains to be fully understood. *In vivo* studies comparing the metabolic profile of G12D cancers with tumors driven by different KRAS variants in **autochthonous** models will be crucial in teasing apart common and disparate metabolic networks. These studies will also provide valuable insights on the contributions of co-mutations, tumor microenvironment and tissue of origin; all factors likely to also influence the metabolic landscape of KRAS-driven tumors.

KRAS-G12D inhibitors

Design of KRAS-G12D inhibitors

The development of allele-specific KRAS-G12C inhibitors demonstrated successful targeting of the previously undruggable KRAS, spearheading the development of allele-specific and pan-KRAS inhibitors. Targeting G12C was made possible by the ability to use a reactive warhead to form an irreversible covalent bond with the mutant cystine [49,50] (Figure 2B). In contrast, G12D inhibitors were developed using alternative approaches due to the inability to directly target the less-reactive mutant aspartate in the G12D protein. MRTX1133 (Mirati) [51] was identified as a noncovalent inhibitor that binds to the switch II pocket of KRAS-G12D, preventing nucleotide exchange and binding of the effector RAF (Figure 2B). It utilizes a piperazinyl group to form an ionic bond with the mutant aspartate, and despite the lack of a covalent bond, it exhibits high anti-cancer properties and will likely enter clinical trials in 2023. In contrast, RMC-9805 (Revolution Medicines) first forms a noncovalent bond between KRAS-G12D and cyclophilin A that then allows the 'cool' nonreactive covalent 'warhead' to slowly bind to the mutant aspartate. RMC-9805 is on-track to enter clinical trials mid-2023. Other KRAS-G12D inhibitors include HRS-4642 (Jiangsu Hengrui Medicine) in Phase 1 clinical trials in China (NCT05533463), as well as TH-Z835 (Tsinghua University) [52], BI-KRASG12D (Boehringer Ingelheim), JAB-22000 (Jacobio) and ERAS-4 (Erasca) in preclinical development. There is also a KRAS-G12D degrader ASP3082 (Astellas), which binds KRAS-G12D to a E3 Ligase to degrade the protein and is currently in Phase 1 clinical trials (NCT05382559).

As KRAS shuttles from a GDP-bound OFF state to a GTP-bound ON state, KRAS inhibitors differ in their abilities to bind these two states. Unlike all other mutants that reduce intrinsic KRAS GTPase activity, G12C exhibits wild-type intrinsic GTPase activity [11]. G12C inhibitors sotorasib [34] and adagrasib [33] function as OFF state inhibitors, binding to GDP-bound KRAS-G12C. In contrast, G12D exhibits a higher inhibition of intrinsic GTPase activity [11]. However, the efficacy of MRTX1133, a primarily OFF state inhibitor, adds support that G12D is in the OFF state for enough time. Significant effort has gone into the designing of inhibitors that bind the ON state of KRAS-G12D, with RMC-9805 binding ON state, and BI-KRASG12D and TH-Z835 binding to both ON and OFF states of KRAS-G12D.

While no clinical data currently exists, KRAS-G12D inhibitors have been tested in pre-clinical models. MRTX1133 has shown strong anti-cancer properties in G12D-driven lung, pancreatic and colorectal adenocarcinoma **syngeneic transplantation** and **xenograft** models [44,53]. MRTX1133 has also been shown to synergize with EGFR inhibition in pancreatic and colorectal adenocarcinoma xenograft models [53], and across a panel of human lung/pancreatic/colorectal adenocarcinoma cell lines with AKT but not MEK/ERK inhibitors [53]. In addition, TH-Z835 has

been shown to synergize with anti-PD1 therapy in syngeneic transplantation models of pancreatic adenocarcinoma [52].

Efficacy of KRAS inhibitors

Therapies targeting oncogenic drivers EGFR or ALK in NSCLC patients achieve response rates of 70–90% as monotherapies [54]. In contrast, KRAS-mutant cancers are not as addicted to oncogenic signaling as EGFR- or ALK-driven cancers, and this dependency differs across tumors of a cancer type as well as across cancer types. Consequently, the monotherapy outcomes for sotorasib [34] and adagrasib [33] in KRAS-G12C NSCLC patients have shown a modest response rate of only 30%. Within this subset, most patients achieve stable disease, with few patients experiencing tumor shrinkage. Additionally, the duration of treatment efficacy is limited and characterized by short-term benefits. For CRC, the response rates are even poorer at 7–19% [55,56]. Critically, biomarkers predictive of durable response, including concurrent mutations, are still largely unknown. Combination therapies are now being trialed (Box 3), with preliminary results from EGFR inhibition for CRC patients [56], and anti-PD-1 therapy for NSCLC patients (NCT04613596) in combination with adagrasib showing promising results.

The recent development and implementation of KRAS-G12C inhibitors demonstrate that this is just the beginning; with more potent KRAS inhibitors and/or effective combination therapies needed to unlock the full potential of KRAS inhibition in clinical settings. Given the poor response

Box 3. Combination therapies using G12C inhibitors

KRAS-G12C mutations occur in 13% of NSCLC and 1–3% of colorectal cancer (CRC) or other patients [55]. The first G12C inhibitors sotorasib and adagrasib entered clinical trials in late 2020, with initial results as a monotherapy in heavily pretreated G12C-mutant NSCLC and CRC patients showing objective response rates of ~35% [33,34] and 7–19%, respectively [55,56]. Since then, trials of combination therapies are ongoing, with some highlighted below.

SHP-2 inhibitors

SHP-2 is a protein tyrosine phosphatase that acts upstream of KRAS. Inhibition of SHP-2 decreases the levels of KRAS-GTP and increases levels of KRAS-GDP that is targeted by KRAS OFF inhibitors, increasing their efficacy [61–63]. In preclinical and cell line models of pancreatic adenocarcinoma, NSCLC, and CRC, the SHP-2 inhibitor SHP099 led to increased response to KRAS-G12C inhibitors *in vitro* and *in vivo* [61–63]. Combination clinical trials of KRAS-G12C inhibitor sotorasib with SHP-2 inhibitor RMC-4630 (NCT05054725) or BBP-398 (NCT05480865) in patients with G12C mutations are ongoing.

EGFR antibodies

EGFR is a receptor tyrosine kinase that acts upstream of KRAS and increases KRAS signaling. Compared with KRAS-G12C NSCLC cell lines, KRAS-G12C CRC cell lines have been shown to have increased EGFR signaling, which increases the GTP-bound state of KRAS and decreases the efficacy of KRAS-G12C inhibition [73]. Notably, the combination of sotorasib with cetuximab, an anti-EGFR monoclonal antibody, resulted in a 46% objective response rate in KRAS-G12C CRC patients [56]. KRAS-G12C inhibitor adagrasib with cetuximab is currently ongoing (NCT04793958). The combination of sotorasib and a pan- receptor tyrosine kinase inhibitor tarloxotinib (targets EGFR, HER2 and HER4) (NCT05313009), or EGFR antibody panitumumab (NCT04185883) is ongoing for KRAS-G12C NSCLC.

Immunotherapy

Treatment with pembrolizumab (anti-PD1 antibody) and sotorasib concurrently resulted in 42-47% of NSCLC patients developing grade 3 or 4 liver toxicity (CodeBreaK100/101; NCT03600883, NCT04185883). However, more promising data has recently been shown for adagrasib, where the combination with pembrolizumab was safe, with less than 10% of patients developing grade 3 liver toxicity and response rates of 49–57% (KRYSTAL-7; NCT04613596). This suggests that pairing immunotherapies with KRAS inhibition remains a viable option.

Other

A Phase 2 trial combining sotorasib with platinum-based chemotherapy is currently ongoing (NCT05118854). The CodeBreaK-101 study is assessing the use of sotorasib in combination with MEK, CDK4/6, and mTOR inhibitors (NCT04185883). Aurora kinase A inhibitor VIC-1911 is currently in clinical trial in combination with sotorasib for treatment of KRAS-G12C NSCLC patients (NCT05374538).

rates as monotherapies for sotorasib and adagrasib, we are likely to see the prioritization of trialing combination therapies when G12D inhibitors enter the clinic. The identification of effective combinations may be guided by the results obtained from G12C inhibitors (Box 3). However, it is also anticipated that G12D inhibitors may have distinct combinations that are more suitable for their specific characteristics. For example, PI3K/AKT inhibitors have been shown to be a more efficacious combination with MRTX1133 than MEK/ERK inhibitors [53]. Additionally, the context of the tumor likely affects how a patient responds. Given that KRAS-G12D mutations are common across many cancers, combination therapies need to be designed around current standard-of-care treatments and tailored for individual cancer types. This approach also presents avenues for therapeutics that leverage vulnerabilities associated with genes found co-mutated with KRAS (Figure 3B). For example, inducing STING expression in KRAS-mutant lung adenocarcinoma harboring *STK11* mutations increased expression of PD-L1 and T cell chemoattractants, suggesting that a STING agonist may elicit response to immunotherapy in these tumors [57].

Resistance to KRAS inhibitors

To date, no studies have assessed resistance mechanisms to G12D inhibitors. Resistance to treatment has been observed for G12C inhibitors and may preempt potential resistance mechanisms for G12D inhibitors. For example, increased receptor tyrosine kinase feedback (e.g., EGFR signaling), reducing GDP occupancy, secondary KRAS mutations, or amplification of the KRAS-G12C allele [58–60]. Some of these resistance mechanisms will likely develop for G12D inhibitors, such as increased upstream or downstream KRAS signaling. In contrast, the different ON to OFF cycling rates of the mutants will impact the level of reduced GDP occupancy, and structural conformation changes to circumvent inhibitor binding will inherently be different depending on the KRAS allele being targeted.

Combination therapies are one approach to delay or prevent the development of resistance observed from monotherapy. Due to the lower toxicity profile and its action upstream of KRAS, SHP-2 inhibitors may overcome resistance mechanisms to OFF inhibitors (e.g., decreased GDP occupancy, amplification of mutant allele) [61–63]. The use of ON state KRAS-G12D inhibitors will also assist in overcoming OFF inhibitor resistance, and/or may be used in combination with OFF inhibitors if the modes of action differ. Pan-KRAS inhibitors such as RMC-6236 (Revolution Medicines) may also help combat resistance that develops for allele-specific inhibitors.

Concluding remarks

KRAS-G12D is the most common KRAS mutation in carcinomas. It elicits unique structural and signaling properties, drives a highly immunosuppressive tumor microenvironment and exhibits the most potent oncogenic potential out of all KRAS variants. While there are common features of KRAS-G12D adenocarcinomas, the effects of harboring a G12D mutation are influenced by the tissue of origin and the co-mutations present within the tumor. These factors largely dictate therapeutic responses to current therapies, and treatment with KRAS-G12D inhibitors will need to be based on the tumor type and factor in the genomic landscape of the tumor. Further studies are needed to identify which factors predict response to therapy and which combination therapies will be efficacious for each cancer type (see Outstanding questions). Given the development of allele-specific KRAS inhibitors, studies are also needed that incorporate head-to-head comparisons between KRAS alleles to better characterize the allele specific effects on tumor biology.

Outstanding questions

Why is G12D the most frequently observed KRAS mutation with the highest oncogenic potential in adenocarcinomas?

To what degree are the tumor characteristics inferred from studies using KRAS-G12D mice transferrable to other KRAS mutant alleles?

What has the greatest influence on tumor biology – the microenvironment of the tissue of origin or the co-mutational landscape of the tumor?

What factors/characteristics (e.g., genetic alterations) predict response to KRAS-G12D inhibitor therapy?

What therapies will be efficacious in combination with KRAS-G12D inhibitors?

Will KRAS-G12D inhibitors need to be tailored to specific tumor types and/or genomic landscape of the tumor?

What mechanisms will drive resistance to KRAS-G12D inhibitors?

Can we directly translate clinical findings with KRAS-G12C inhibitors when designing KRAS-G12D inhibitor clinical trials?

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Declaration of interests

No interests are declared.

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