Molecular Biomarkers of K-RAS Dependency

Subjects: Biochemistry & Molecular Biology

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Oncogenic v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-RAS*) plays a key role in the development and maintenance of pancreatic ductal adenocarcinoma (PDAC). The targeting of *K-RAS* would be beneficial to treat tumors whose growth depends on active *K-RAS.* The analysis of *K-RAS* genomic mutations is a clinical routine; however, an emerging question is whether the mutational status is able to identify tumors effectively dependent on *K-RAS* for tailoring targeted therapies. With the emergence of novel *K-RAS* inhibitors in clinical settings, this question is relevant. Several studies support the notion that the *K-RAS* mutation is not a sufficient biomarker deciphering the effective dependency of the tumor. Transcriptomic and metabolomic profiles of tumors, while revealing *K-RAS* signaling complexity and *K-RAS*-driven molecular pathways crucial for PDAC growth, are opening the opportunity to specifically identify *K-RAS*-dependent- or *K-RAS*-independent tumor subtypes by using novel molecular biomarkers. This would help tumor selection aimed at tailoring therapies against *K-RAS*. In this review, we will present studies about how the *K-RAS* mutation can also be interpreted in a state of *K-RAS* dependency, for which it is possible to identify specific K-RAS-driven molecular biomarkers in certain PDAC subtypes, beyond the genomic *K-RAS* mutational status.

Keywords: pancreatic cancer ; KRAS-dependency ; targeted therapy

1. Definition

Activating mutation of the KRAS oncogene occur in 88% cases of pancreatic ductal adenocarcinoma (PDAC) and is the initiating genetic event in PanIN formation (Pancreatic Intraepithelial Neoplasia). The genomic landscape of PDAC shows multiple genetic events, most of them contributing to tumor maintenance in cooperation with the *K-RAS* activation, most likely with a different degree of dependency according to the history of the tumor development, staging, or treatments. Deciphering the effective dependency of the tumor on *K-RAS* or on alternative oncogenes is key to promote targeted therapies in PDAC.

2. Oncogenic K-RAS: A Critical Driver for Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related death with an overall five-year survival rate of only 8% [1][2]. PDAC is diagnosed at an advanced, inoperable stage in the vast majority of cases and most of the patients diagnosed with surgically resectable disease recur within the first 2–3 years after the operation [3]. Current systemic first-line treatment for advanced inoperable PDAC includes polychemotherapy regimens such as folinic acid/ 5-fluorouracil/irinotecan/oxaliplatin,(FOLFIRINOX),cisplatin/nab-paclitaxel/capecitabine/gemcitabine (PAXG), gemcitabine/nab-paclitaxel, and gemcitabine monotherapy in a small sub-group of elderly, frail, or unfit patients. Primary chemoresist/ance or recurrence rates in PDAC remain high, and overall survival from the start of first-line ranges approximately from 8 to 12 months [4][5][6]. Currently, no validated prognostic or predictive biomarkers exist for PDAC, except for general clinical criteria (performance status, disease burden, CA19.9 levels), and no targeted or immune-based therapies have proven to be effective so far, although a large number of clinical trials are ongoing and efficacy data for novel treatments are awaited [7][8][9].

The RAS pathway is one of the most frequently altered pathways in cancer, found in approximately 19% of all human cancer harboring *RAS* gene mutations [10]. Among the three major isoforms of oncogenic *RAS*, *K-RAS* is the most frequently mutated **[11][12][13].** Mutation of *K-RAS* is the initiating genetic event of pancreatic intraepithelial neoplasias (PanINs) and is required to drive PDAC development and tumor maintenance [14][15][16][17][18] Oncogenic mutant *K-RAS* is found in about 88% of PDAC [10]. Oncogenic mutation in *K-RAS* protein leads to aberrant or constitutive signaling even in the absence of growth factors, leading to increased proliferation, invasion, and metastasis [19]. Inactivating mutations in crucially tumor suppressor genes, particularly *CDKN2A/p16*, *TP53*, and *SMAD4*, cooperate with oncogenic *K-RAS* to promote aggressive PDAC tumor growth and metastasis [19][20][21][22][23][24][25][26].

K-RAS is a member of the RAS family of Guanosine Tri-Phosphate(GTP)-ases that regulates several cellular processes including survival, proliferation, differentiation, migration, and apoptosis [27]. RAS proteins function as molecular switches promoting conversion from an inactive to an active GTP-bound state. Though tightly controlled in normal cells, the mutation in *K-RAS* gene leads to constitutive GTP-bound *K-RAS*, rendering constitutively activated RAS protein and determining the persistent activation of downstream signaling pathways resulting in uncontrolled activation of proliferation and survival pathways [28][29][30]. The mutations in *K-RAS* consist of single amino acid substitutions and are predominant at residues G12, G13, and Q61. Oncogenic mutations of G12 or G13 create a steric block that prevents the hydrolysis of GTP, whereas substitutions of Q61 interfere with the coordination of a water molecule required for GTP hydrolysis; these point mutations lead to a prevalence of the GTP-bound state and to the constitutive activation of *K-RAS* [19].

Once in its active form, *K-RAS* engages complex and dynamic downstream effectors such as the RAF/MEK and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. The Mitogen-Activated Protein Kinase (MAPK) pathway is a key mediator of oncogenic *K-RAS* signaling and BRAF is the principal mediator of MAPK signaling in *K-RAS* dependent cancer growth. The BRAF V600E mutations are mutually exclusive with *K-RAS* mutations [31]. However, genetic studies in mice models revealed that *BRAF* ^{V600E} mutation is sufficient to induce PanIN formation in the pancreas of *K-RAS* wild-type (WT) mice, and to develop lethal PDAC when combined with a *TP53* mutation [32]. The PI3K-dependent pathway drives tumor growth and cooperates with oncogenic K-RAS to develop PDAC [33][34]. The major driver mutations in this pathway that promote pancreatic tumor development include mutations in the catalytic and regulatory PI3K subunit, amplification of the PI3K downstream effector AKT2, and deletion/loss of tumor suppressor Phosphatase/TENsin homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K/AKT signaling [35][36][37].

It is important to mention that a relatively large proportion of patients with PDAC display germline mutations of some DNA damage repair (DDR) genes. Specifically, 18% of PDAC harbor mutations in homologous recombination (HR) DDR pathways such as *BRCA1* and *BRCA2* [38][39], and the BRCA2 inactivation in combination with p53 deficiency promotes *K-RAS* driven PDAC development [40][41].

3. Defining the K-RAS Dependency in PDAC

K-RAS mutation represents a common genetic event in PDAC, being mutated in almost 88% of cases [42]. However, contrary to preclinical studies, clinical approaches have demonstrated poor efficacy of treatments targeting the *K-RAS* pathway in PDAC tumors carrying a *K-RAS* mutation. One of the potential explanations is the possibility that the genomic *K-RAS* mutation is not an efficacious molecular determinant for tumor dependency on *K-RAS* activation. Indeed, the absence of *K-RAS* gene mutations does not always correlate with *K-RAS* pathway inactivity due to the activation of the other components of the network [43][44], and conversely, the presence of *RAS* mutations does not necessarily predict for dependency. This can depend on the activation of additional active molecular pathways that can complement or subside for *K-RAS* activation. Thus, determining the genomic mutational status of specific genes is not always beneficial for predicting pathway activation and the drug response with targeted compounds [45].

Assessing *K-RAS* pathway activation status by more comprehensive methods will help better predict the *K-RAS* dependency of tumors. Years have passed since the concept of oncogene addiction was first proposed, linking single dominant oncogene to tumor growth and survival [46]. Omics studies, such as genomics, transcriptomics, and metabolomics can lead to extensive molecular profiles, which act as tools to reevaluate the traditional definition of addiction and oncogene dependency as a functional definition based on the oncogene-driven phenotype, regardless of the presence or not of a specific oncogenic gene mutation. A large number of observations in animal models and pancreatic cancer cell lines revealed that the *K-RAS* gene, although mutated or overexpressed, is dispensable in a subset of human and mouse *K-RAS* mutant PDAC cell lines. By using RNA interference, inducible transgenic models or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 technology, it has been possible to classify two subtypes of PDACs harboring the *K-RAS* mutation: tumors in which a *K-RAS* depletion led to apoptosis and thus they are considered as "*K-RAS*-dependent" and others that are resistant to *K-RAS* depletion, without a sign of apoptosis, and considered as "*K-RAS*-independent" [47][48][49][50][51][52][52]. The extensive molecular characterization of such models shed a light on additional features that would be missed based on simple genomic classification of the tumor, with the potential of a profound implication from a therapeutic and prognostic point of view.

The goal of this review is to provide an overview of emerging molecular markers of *K-RAS* oncogene dependency, regardless of the genomic mutation status. Gene expression profile studies, in particular, allow to understand if the *K-RAS* pathway could be activated by mutations of the *K-RAS* gene or by many other mechanisms, and they help to deconstruct the *K-RAS* network contribution in tumor progression [45][48][53]. In addition, metabolomics studies identified pathways and metabolites that are specifically enriched in *K-RAS*-dependent PDAC to mediate a metabolic reprogramming relevant to tumor growth. Thus, multiple and specific molecular biomarkers underlining the oncogenic phenotype associated with a

real dependency on *K-RAS* oncogene in PDAC are emerging. The translational value of such information is manifold since i) it helps to find novel diagnostic biomarkers that could overcome the limitation of a genomic-based approach for an effective determination of *K-RAS* dependency and ii) it provides the ground for novel therapeutic strategies to define effective targeted therapy against a subclass of PDAC patients, whose tumors have *K-RAS* dependency and actionable vulnerabilities.

In the next paragraphs, we will discuss molecular profiling based on transcriptomic and metabolomics studies that provided novel markers for *K-RAS* dependency in PDAC.

4. Therapeutic Opportunities Against K-RAS-Dependent PDAC

Strategies developed to target *K-RAS* and its downstream effectors are likely to elicit a stronger therapeutic response against *K-RAS*-dependent tumors. Far from exhaustive, this section will provide some examples of these strategies, including direct *K-RAS* inhibitors, inhibitors of plasma membrane association, inhibitors of downstream signaling, and of metabolic phenotypes. The first compounds identified as capable of directly inhibiting mutant *K-RAS* proteins were small molecules able to interfere with the *K-RAS*-Guanosine Diphosphate (GDP) complex and inhibit Son of Sevenless homolog (SOS)-mediated nucleotide exchange [54][55][56][57]; other compounds instead efficiently were able to bind to RAS-GTP, thus inhibiting signaling cascades downstream of *K-RAS.* However, these compounds have not yet been investigated in clinical settings [58][59]. The targeting of enzymes involved in the post-translational modifications of *K-RAS*, necessary for protein activation, has been also investigated. Farnesylation is a post-translational modification crucial for the proper plasma membrane localization of *K-RAS* and downstream pathways activation. In this context, a farnesyltransferase inhibitor (FTI) termed tipifarnib was developed as a potential inhibitor of *K-RAS* [60]. Moreover, deltarasin, a small molecule that binds the prenyl-binding protein PDEδ, that is crucial for plasma membrane localization of farnesylated *K-RAS*, has also been developed [61][62]. However, clinical trials did not show a significant anti-tumor effect and any survival benefit for patients [63][64].

Current efforts to block activated *K-RAS* are also focused on downstream *K-RAS*-dependent pathways. One of the commonly studied pathways is the RAF-MEK-ERK pathway, and several MEK inhibitors have been developed including trametinib and selumetinib [65][66]. Clinical trials' results related to these inhibitors failed to show clinical benefit and effect on survival in patients [67][68]. However, a few phase I/II studies are underway to test the efficacy of other MEK inhibitors including pimasertib and refametinib in combination with gemcitabine [66][69][70]. Several small molecules have been developed to target PI3K-, AKT-, and/or the Mammalian Target Of Rapamycin (mTOR)-dependent pathway, but monotherapies with PI3K-dependent pathway inhibitors alone failed to show efficacy in *K-RAS*-mutant cancers [71]. However, the combination of PI3K with RAF-MEK-ERK inhibitors exhibited potent tumor growth inhibitory activity [72], but clinical results do not match with those seen in preclinical models [73]. Importantly, in most of the clinical studies cited above, the assessment for *K-RAS* dependency has not been performed before treatments, thus therapies were not tailored for the patients' population which were highly likely to respond.

Recently, preclinical evidence revealed a specific covalent inhibitor with high selectivity for *K-RAS*^{G12C} able to trap the inactive *K-RAS*-GDP complex, thus blocking nucleotide exchange and RAS downstream signaling [74]. Currently, the agent is being evaluated in a phase I/II clinical trial (NCT03600883) for patients with advanced solid tumors harboring a *K-RAS*^{G12C} mutation. Nonetheless, G12C mutations are rarely observed in PDAC (1%), and similar approaches targeting *K-RAS*^{G12D} and *K-RAS*^{G12V} mutations, which constitute the prevalent *K-RAS* mutations in PDAC are needed [19].

Autophagy and macropinocytosis are both biological mechanisms that contribute to the growth and survival of K-RAS mutant pancreatic cancer cells [75][76], and clinical studies are evaluating hydroxychloroquine as autophagy inhibitors in combination with other chemotherapeutic drugs [77].

Finally, the dependency on pyrimidine metabolism in *K-RAS*-dependent PDAC has been exploited in preclinical models by Mottini et al. [52]. Thanks to a computational drug repositioning approach using *K-RAS*-driven signatures, authors repurposed 5-aza-2'-deoxycytidine (decitabine), an FDA-approved drug, to inhibit *K-RAS*-dependent PDAC tumor growth. *K-RAS*-dependent PDACs were highly sensitive to decitabine treatment, showing reduced cell viability and impaired tumor growth. On the contrary, decitabine treatment in K-RAS-independent cell lines and tumors did show minimal or no effect.

In conclusion, several therapeutics have been developed especially for treating *K-RAS*-driven PDAC and tested in preclinical or clinical settings. However, in most cases, *K-RAS* dependency has not been assessed on the treated population, and the response rate upon treatments has not been evaluated on the basis of the effective *K-RAS* dependency of tumors. Based on the emergency of biomarkers for *K-RAS* dependency, as described in this review, the results of clinical trials and drug effectiveness should be reevaluated for a complete assessment of drug efficacy in PDAC.

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