Cancer Biol Med 2019. doi: 10.20892/j.issn.2095-3941.2018.0530

REVIEW

From basic researches to new achievements in therapeutic strategies of KRAS-driven cancers

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ABSTRACT Among the numerous oncogenes involved in human cancers, KRAS represents the most studied and best characterized cancer-related genes. Several therapeutic strategies targeting oncogenic KRAS (KRAS^{onc}) signaling pathways have been suggested, including the inhibition of synthetic lethal interactions, direct inhibition of KRAS^{onc} itself, blockade of downstream KRAS^{onc} effectors, prevention of post-translational KRAS^{onc} modifications, inhibition of the induced stem cell-like program, targeting of metabolic peculiarities, stimulation of the immune system, inhibition of inflammation, blockade of upstream signaling pathways, targeted RNA replacement, and oncogene-induced senescence. Despite intensive and continuous efforts, KRAS^{onc} remains an elusive target for cancer therapy. To highlight the progress to date, this review covers a collection of studies on therapeutic strategies for KRAS published from 1995 to date. An overview of the path of progress from earlier to more recent insights highlight novel opportunities for clinical development towards KRAS^{onc}-signaling targeted therapeutics.

KEYWORDS Direct inhibition; downstream effectors; oncogenic KRAS; drug target sites; small GTPases; signal transduction; targeting synthetic, lethal interactions; therapeutic strategies

Introduction

KRAS is a small GDP/GTP-binding protein that transduce extracellular signals into intracellular responses. It cycles between an inactive, GDP-bound ("off") state and an active, GTP-bound ("on") state. This off/on cycle is tightly regulated by RAS-specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)[1]. In its active state, KRAS binds and activates various effector proteins function and thus regulate downstream signaling pathways (**Figure 1**). The conserved GDP/GTP-binding (G) domain of KRAS contains two flexible regions, the switch regions 1 and 2, which provide a functional platform for the interaction with regulators and effectors[2-4]. The C-terminus of KRAS, which is highly variable among the RAS paralogs, is the site for post-translational modifications and responsible for KRAS anchorage to the plasma membrane[5-7].

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Received January 18, 2019; accepted June 10, 2019.
Available at www.cancerbiomed.org

Upstream signaling pathways of KRAS are activated by binding of ligands to their transmembrane receptors, mostly receptor tyrosine kinases, and recruitment of docking proteins, such as GRB2, in complex with RAS-specific GEFs, which facilitates KRAS activation (**Figure 1**)[8-10]. GTP-bound KRAS further transduces the signal to its downstream effectors and thus activates multiple signaling pathways [11-15]. Thereby, KRAS controls various cellular processes, including survival, growth, proliferation, differentiation, and apoptosis [16-18].

With the discovery of the mutational activation of *RAS* genes in human cancers dating back to the 1960s, extensive studies have been conducted to understand the localization, regulation and signaling of RAS proteins with the ultimate goal of developing anti-RAS drugs for cancer treatment[3]. Somatic mutations, most frequently identified *KRAS4B* (oncogenic *KRAS* or *KRAS^{onc}*) (COSMIC), contribute to robust gain-of-function effects and to various types of cancers as well as leukemia and lymphoma tumors[19-22]. Due to reduced GTP hydrolysis and resistance to GAPs[19,20], KRAS^{onc} persist in a constitutive active state and thus, strongly contribute to neoplastic signal transduction[23].

Despite intensive efforts on the understanding of the

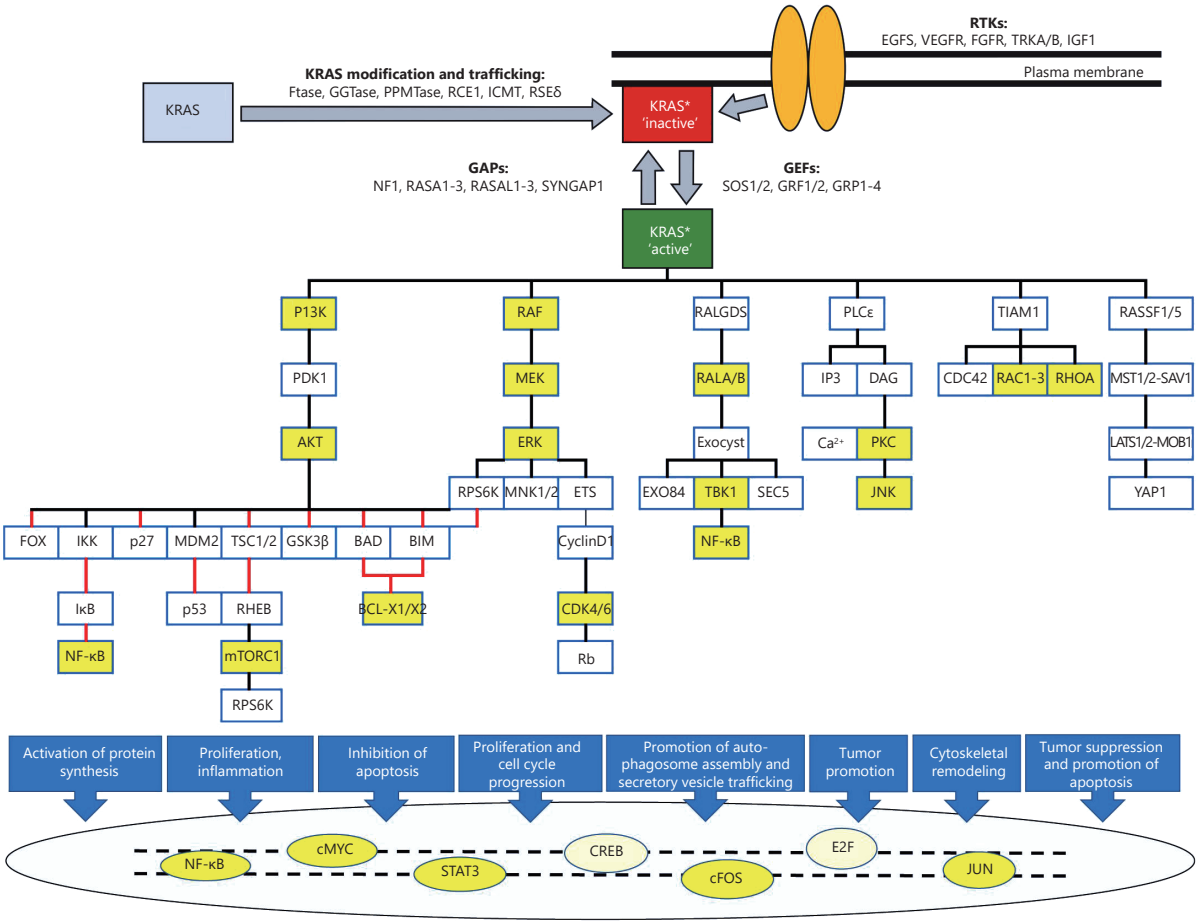

Figure 1 KRAS signaling pathways. Different upstream RTKs, regulators (GEFs and GAPs), downstream effectors, and transcription factors are presented along with posttranslational modification of newly synthesized KRAS (gray box) to trafficking modified KRAS (red box) and its association with plasma membrane. Stimulatory effects are shown in black lines and inhibitory effects in red lines. The color yellow shows some of the downstream therapeutic targets mentioned in this article. The asterisk * highlights posttranslationally modified KRAS.

mechanisms of intracellular trafficking, regulation and signaling activity of RAS proteins, specific inhibition of oncogenic RAS has not been clinically established to date[3]. Among the RAS protein family, *KRAS* mutations are the most common oncogenic driver in many human cancers[4]. Additionally, KRAS^{onc} is a strong predictive biomarker of resistance to anti-EGFR (Epidermal Growth Factor Receptor) treatment. Therefore, the prevalence of *KRAS* mutations in a number of human cancers and its inherent resistance to anti-EGFR targeting underscores the clinical relevance of targeting KRAS^{onc} in cancer treatment[2,24].

Extensive research on different cell lines harboring the *KRAS* mutation have been conducted, including a pancreatic cancer cell line (PANC-1)[25], human colorectal cancer cell lines (DLD-1, HCT-116, and Colo-320 cells)[26], non-small cell lung cancer (H441 cells)[27], human bronchial epithelial cells (HBEC3KT cells)[28], human alveolar basal epithelial cells (A-549 cells)[29], human oral squamous cell carcinoma (H157 cells)[29], human breast adenocarcinoma cells (MCF-7 and SKBR3-LR cells)[30], murine embryonic fibroblasts (MEFs)[31], and acute myeloid leukemia cells (NOMO-1)[25]. According to studies on targeting the *KRAS* oncogene, therapeutic strategies can be divided into two main categories: 1) small molecule inhibitors, which are synthetically lethal to mutant *KRAS* or designed to prevent the post-translational processing of KRAS^{onc}, upstream pathways, KRAS^{onc}/GEF interactions and downstream KRAS* effectors; and 2) anti-KRAS genetic therapies, which interfere with the expression of *KRAS* or other components of KRAS^{onc}-associated signaling pathways.

The complexity of KRAS signaling pathways, in which KRAS protein interacts with many different upstream mediators, downstream effectors, and transcription factors in a nonlinear fashion, has a critical role in the lack of effective

treatment[32-34]. Thus, a better understanding of KRAS interactions with other proteins and transcription factors may provide new opportunities for effective treatment (**Figure 1**).

In this review, we provide a snapshot view of the rich history of KRAS research by chronologically discussing representative key retrospective discoveries regarding the various therapeutic options for cancers associated with *KRAS* mutations. In addition to basic original anti-KRAS^{onc} therapeutic mechanisms, novel approaches, including inhibition of the embryonic stem cell-like program[18], targeting of upstream tyrosine kinases[10], stabilization of KRAS^{onc} G-quadruplex structures[35], inhibition of inflammation[36], and targeting of metabolic peculiarities[37], for suppression of aberrant *KRAS* activation in cancers are also explained (**Figure 2**).

In addition to *KRAS* mutations, amplification of wild-type *KRAS* gene or EGFR mutation leads to the over-expression or over-activation of KRAS, respectively. Some studies have shown that both over-expressed *KRAS* and *KRAS*^{onc} can be associated with aggressive and metastatic cancer phenotype[38,39]. Regarding these similarities, some of the targeting strategies discussed in this review may be applied for both KRAS and KRAS^{onc}, *e.g.*, inhibition of downstream signaling pathways or inhibition of plasma membrane localization. In contrast, structural differences between KRAS^{onc} and KRAS provide distinct therapeutic opportunities[40]. Some studies, which are referred to in this review, focus on total RAS proteins. Considering that the *KRAS* mutation represents approximately 90% of identified *RAS* mutations[33], the results of studies on total RAS proteins could certainly be applied to KRAS protein.

Inhibition of KRAS localization

KRAS localization in the plasma membrane is a critical step

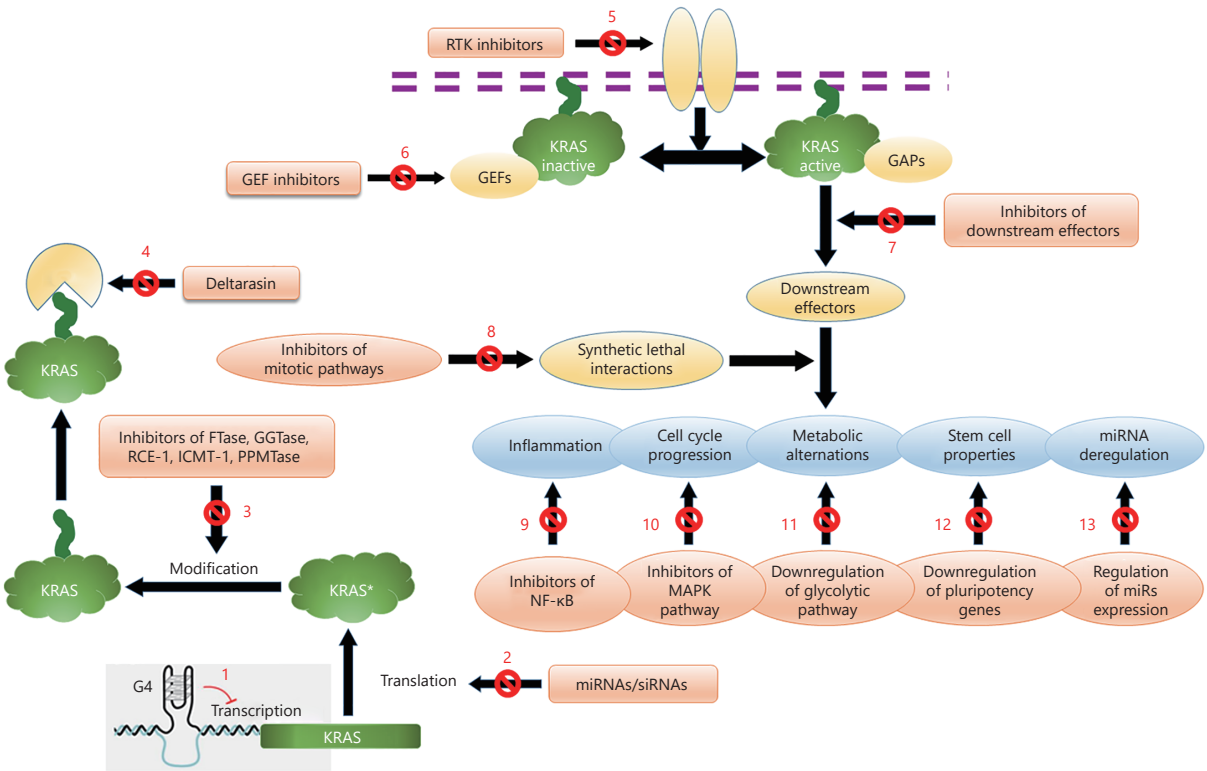

Figure 2 Different therapeutic targets for KRAS driven cancers. The most important of these therapeutic strategies discussed in this article are shown by numbers: (1) Inhibition of transcription by G4 elements. (2) Inhibition of translation through complementary microRNAs. (3) Targeting enzymes posttranslationally modifying KRAS. (4) Targeting KRAS membrane trafficking. (5) Interference with upstream signaling by targeting of receptor tyrosine kinases. (6) Targeting GEFs and RAS activation. (7) Targeting KRAS effectors and downstream signaling pathways. (8) Suppression of synthetic lethal interactions. (9) Targeting inflammatory signaling pathways. (10) Targeting cell cycle progression. (11) Reregulation of metabolic alternations. (12) Reprogramming of stem cell properties. (13) Upregulation of miRs with anti-KRAS activity. Black arrows with blocked red circles are referred to inhibited targets as potential therapeutic approaches.

for its activation and signaling[41]. Thus, inhibition of KRAS localization provides new insights for cancer treatment. There are three main approaches to prevent KRAS^{onc} localization: 1) inhibition of KRAS^{onc} post-translational modifications, 2) displacement of KRAS^{onc} from the membrane, and 3) impairment of proper KRAS^{onc} intracellular trafficking[41-43]. After translation of KRAS protein, it must undergo a series of post-translational modifications, which facilitate its association with the cell membrane.

Initially, the enzyme farnesyl transferase (FTase) catalyzes the addition of a farnesyl isoprenoid moiety to the thiol group of the terminal cysteine in the CAAX motif of KRAS protein[44]. CAAX stands for C, a cysteine, A for aliphatic amino acids and X for any amino acid. Next, protease RAS-converting enzyme-1 (RCE-1) cleaves the terminal AAX amino acids, and then the carboxyl group of the cysteine is methylated by isoprenyl-cysteine carboxymethyl transferase-1 (ICMT-1)[43,45,46]. Multistep post-translational modifications of KRAS protein provide several possible drug targets, including FTase, RCE-1, and ICMT-1[43,44]. Thus, attempts have been made to target KRAS post-translational modifications to inhibit its membrane localization and thus its activation and downstream signaling for the treatment of cancers. Prevention of KRAS^{onc} processing to form a stable interaction with the cell membrane is not the only mechanism to reduce the population of KRAS^{onc} at the membrane. Displacement of KRAS^{onc} from the membrane and the impairment of proper trafficking are the two other strategies[47,48]. For instance, perturbation of the subcellular distribution of phosphatidylserine leads to a significant reduction of electrostatic interactions between KRAS^{onc} and the plasma membrane, resulting in its displacement from the membrane. Another strategy triggering the mislocalization of KRAS^{onc} is phosphorylation of S181 in the C-terminal hypervariable region (HVR) of KRAS^{onc}, thereby activating the farnesyl-electrostatic switch.

Targeting post-translational modifications of KRAS^{onc} to inhibit its plasma membrane localization appeared to be promising in preclinical studies; however, alternative post-translational modifications of KRAS^{onc} and disruption of the prenylation of proteins other than KRAS^{onc} have led to disappointing results. In spite of the earlier unsuccessful results, continuation of the studies on the disruption of KRAS^{onc} plasma membrane localization has led to the development of novel treatment outcomes. For example, RAS binding proteins, such as phosphodiesterase delta subunit (PDEδ), have attracted considerable attention as a new target[42,49]. Prenylation of KRAS increases its hydrophobicity and, thus, reduces its solubility. PDEδ facilitates the distribution of RAS family proteins by covering hydrophobic group. Therefore, inhibition of the RAS-PDEδ interaction prevents oncogenic RAS (RAS^{onc}) activation and signaling and results in an anti-cancer effects on RAS-transformed cells. In recent studies, blockade of the prenyl-binding pocket of PDEδ demonstrated promising result[42,49]. In order to have a view on the progress has been made for disruption of KRAS^{onc} plasma membrane localization, studies examining the blockade of KRAS^{onc} processing, mislocalization, and trafficking published from 1993 to 2016 are summarized chronologically (**Table 1**).

Direct inhibition of KRAS^{onc}

In response to extracellular stimuli that activate cell surface receptors, RAS protein members mediate the transduction of extracellular signals to intracellular responses. Small GTPases of the RAS family function as molecular switches that cycle between active, GTP-bound and inactive, GDP-bound states[66]. Activation of upstream signaling pathways results in the recruitment of GEFs, such as SOS1 and SOS2, which facilitate KRAS activation by catalyzing the release of GDP from KRAS[67,68]. Activated KRAS controls different cellular processes that are also involved in the transformation of normal cells to the malignant phenotype[69].

The intrinsic GTPase activity of wild-type KRAS is enhanced by GAPs; however, oncogenic *KRAS* mutations lead to the impairment of GTP hydrolysis and cause GAP insensitivity and thus constitutive activation of KRAS^{onc}[70-73]. Indeed, inhibition of the constitutively active KRAS^{onc} is a conceptually ideal strategy for cancer therapy. Two general mechanisms have been suggested for direct inhibition of RAS proteins, including decreasing the proportion of KRAS^{onc} in its GTP state and disrupting the KRAS^{onc}-effector interactions. To decrease KRAS^{onc}-GTP levels, several approaches have been used, such as the inactivation of KRAS^{onc} with small molecules or GTP analogs that facilitate GTP hydrolysis activity, interference with the nucleotide exchange process through disruption of the SOS-KRAS^{onc} interaction, subversion of the native nucleotide preference of the KRAS^{onc} to favor GDP over GTP, irreversible inhibition of the KRAS^{onc} with its covalent modification, inactivation of KRAS^{onc} in the GTP state, inhibition of intrinsic nucleotide exchange, and inhibition of nucleotide binding[40,67,74].

Activation of downstream effectors, such as RAF kinases, is accomplished through direct interaction of KRAS^{onc} with its effectors. Likewise, other approaches in treatment of KRAS^{onc}-driven cancers, first generation of RAF kinase

inhibitors had limited clinical benefit where the inhibitors found to paradoxically activate ERK pathway through the induction of RAF dimerization in RAS-mutant cancers[75]. Discovery programs in the development of new RAF

Table 1 Inhibition of RAS plasma membrane localization

Strategy	Target	Inhibitor	Result	RAS type	Cells/tissues	Reference
Inhibition of post-translational modification	Ftase	FTI-277	Inhibition of oncogenic HRAS and KRAS processing and PM localization with blocking constitutive activation of MAPK	KRAS and HRAS	NIH3T3 fibroblasts	[50]
	Prenylated protein methyltransferase (PPMtase)	S-trans,trans-farnesylthiosalicylic acid	Inhibition of cell growth	HRAS	Rat1 fibroblasts	[51]
	Ftase	B956	Inhibition of human tumor xenograft growth	KRAS	Colon carcinoma	[52]
	Ftase	Lonafarnib (SCH-66336)	Inhibition of soft agar and human tumor xenograft growth	HRAS and KRAS	NIH3T3 and lung carcinoma	[53]
	Ftase	Lonafarnib (SCH-66336)	Inhibition of colony formation of tumor cells	KRAS	Colon and pancreatic cancer	[54]
	Ftase and GGTase	FTI-277 and GGTI-297	Inhibition of tumor growth	KRAS	NIH3T3 and lung carcinoma	[55]
	Ftase	Lonafarnib (SCH-66336)	Cell cycle arrest in G2 to M phase (KRAS mutated cells) and in G1 phase (HRAS mutated cells)	KRAS and HRAS	Lung, colon, pancreas, and NIH3T3	[56]
	Ftase	BMS-214662	Inhibition of growth attributed to the induction of apoptosis and curative response in human tumor xenografts	HRAS	Colon carcinoma	[57]
	Ftase	L-744, 832	Promotion of apoptosis and cell cycle arrest lead to inhibition of anchorage-dependent growth	HRAS and NRAS	Pancreatic cancer	[58]
	Ftase	FTI-2153	Accumulation of cells in prometaphase by blocking bipolar spindle formation and chromosome alignment	HRAS	Lung cancer	[59]
	RCE-1	Creadenovirus excision of RCE-1	Reduction of cell growth and RAS-induced transformation	KRAS	Primary mouse embryonic fibroblasts and skin carcinoma	[45]
	ICMT-1	Methotrexate	Decrease in RAS methylation, mislocalization of RAS, and decreased phosphorylation of MAPK and AKT	KRAS, NRAS, and HRAS	Colon cancer	[60]

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Continued

Strategy	Target	Inhibitor	Result	RAS type	Cells/tissues	Reference
	ICMT-1	Knockout of ICMT	*In vitro* and *in vivo* inhibition of cell growth and oncogenic transformation	KRAS	Primary mouse embryonic fibroblasts	[45]
	ICMT-1	Cysmethynil	Mislocalization of RAS and impaired epidermal growth factor signaling lead to blocking of anchorage-independent growth	KRAS, NRAS, and HRAS	Mouse embryonic fibroblast	[61]
	ICMT-1	Knockout of ICMT	*In vivo* reduction of splenomegaly, immature myeloid cells in peripheral blood, and tissue infiltration by myeloid cells	KRAS	Myeloproliferative disorder	[46]
	Ftase and GGTase	Allel knockout	Significant reduction in lung tumors and improved survival without apparent pulmonary toxicity	KRAS	Lung cancer	[44]
	ICMT-1	Cysmethynil or inhibitory RNA	Marked inhibition of tumor growth results from autophagy-induced apoptosis	Unknown	Liver and mouse embryonic fibroblast	[43]
Displace RAS from plasma membrane	Membrane-bound farnesyl-binding proteins	Salirasib	Reduction of the amount of RAS, disruption of serum-dependent and epidermal growth factor-stimulated ERK activation, inhibition of both anchorage-dependent and anchorag- independent growth, inhibition of tumor growth xenograft	KRAS	Pancreatic cancer	[62]
	Membrane-bound farnesyl-binding proteins	Bryostatin-1	Phosphorylation of KRAS and its dissociation, promotion of apoptosis, and reduction of *in vitro* and *in vivo* cell growth	KRAS	Jurkat T cells and NIH 3T3 cells	[63]
	Membrane-bound farnesyl-binding proteins	Salirasib and Gemcitabine	Tumor growth inhibition among xenografts, reduction of KRAS, pAKT, and pMAPK, and decrease in total RAS level of liver biopsies	KRAS	Pancreatic cancer	[47]
	Plasma membrane	Fendiline	Redistribution of KRAS from plasma membrane and inhibition of downstream signaling pathways	KRAS	Pancreatic, endometrial, lung, and colon cancer	[41,48]

Continued

Continued

Strategy	Target	Inhibitor	Result	RAS type	Cells/tissues	Reference
	Plasma membrane	Metformin	Inhibition of cell proliferation, MAPK activation, and induction of apoptosis	KRAS	Pancreatic, colon, lung, and endometrial cancer	41,64
	Plasma membrane	Staurosporine and analogs	Perturbation of phosphatidylserine subcellular distribution leads to significant decrease of cell proliferation and MAPK signaling	KRAS	Madine-Darby Canine Kidney cells (MDCK)	41,65
Interfering in proper RAS trafficking	PDEδ	Benzimidazole compounds (Deltarasin)	*In vitro* and *in vivo* inhibition of cell proliferation and reduced activity of ERK	KRAS	Pancreatic cancer	49
	PDEδ	Pyrazolopyridazinones	Inhibition of KRAS-PDEδ interaction, reduction of cell proliferation, reduced signaling through ERK and S6P	KRAS	Pancreatic cancer	42

inhibitor compounds overcome limitations associated with RAF dimerization. Next generation inhibitors take two approaches to combat RAF dimerization. The first approach is the development of compounds with the equal potency for inhibition of both monomeric and dimeric RAF. The second strategy is the recruitment of ATP binding cleft to disrupt RAF dimerization[75]. Other than these therapeutic strategies, progress has been made in generating alternative agents to inhibit KRAS^{onc}-RAF interaction which is needed to stimulate RAS-dependent oncogenic signaling[40,76]. Thus, a better understanding of the detailed interactions of KRAS^{onc} with RAS binding domains and RAS association domains of its downstream effectors provides alternative opportunities for the inhibition of intermolecular interactions[77,78]. **Table 2** provides a summary of studies examining the direct inhibition of KRAS mutant from 1997 to 2017.

Direct inhibition of KRAS^{onc} probably one of the most important therapeutic strategies, has some drawbacks. Direct targeting of this oncogene is difficult owing to its picomolar affinity for GTP/GDP. Furthermore, the interaction of KRAS with small molecules that facilitate GTP hydrolysis is challenging because the active site is occupied by guanine nucleotides, and there is little space for binding small molecules[94]. KRAS molecular switching and signaling are accomplished by protein-protein interactions. Inhibition of these interactions requires a detailed understanding of the interacting interfaces and their characteristics. Additionally, the relative featureless topologies of these surfaces and poor drug-like properties of peptides that disrupt protein-protein interactions make the inhibition more challenging[73]. While targeted therapy against many cancers, such as EGFR-mutated cancers, provides effective responses, no FDA-approved KRAS^{onc}-targeted therapy is currently available, and cytotoxic chemotherapy remains the best option for patients with KRAS^{onc}-driven cancers. Hopefully, following the earlier failures in the direct inhibition of KRAS^{onc}, a new wave of research in recent years has provided promising results. The KRAS oncoprotein has some specific structural features in comparison to wild-type KRAS. Selective targeting of these differences allows direct inhibition of the KRAS mutant without affecting wild type KRAS. For example, recent studies focusing on the *KRAS*-G12C mutation as a direct inhibition strategy have been showed significant results. In this type of mutation, the thiol group of the cysteine residue located close to the nucleotide-binding pocket, switch I, and switch II, are targeted by different small molecules that result in the inhibition of downstream interactions. Since *KRAS*-G12C is the most common mutation in lung cancer patients, the translation of this agent to clinical practice would be a significant approach for generating novel anti-KRAS^{onc} therapeutics[40,67].

RNA interference

The KRAS oncogene activates multiple downstream cellular pathways to drive the progression of cancer[1,95]. Because of

Table 2 Direct inhibition of KRAS mutant as therapeutic strategy

General mechanism of inhibition	Specific mechanism of inhibition	Inhibitor	Result	Reference
Decreasing the proportion of RAS in GTP state	Inhibition of nucleotide exchange process without displacing of GDP	SCH 53870	Inhibition of nerve growth factor -stimulated neurite outgrowth	[79]
	Impairing the nucleotide exchange and acceleration of the RAS GTPase activity	Sulindac sulfide	Decreases the RAS induced activation of the CRAF1 kinase	[80]
	Stimulation of GTPase activity of mutant RAS	GTP analogue [diaminobenzophenone-phosphoroamidate-GTP (DABP-GTP)]	DABP-GTP restore GTPase activity of mutant KRAS	[71]
	Inactivation of KRAS in the GTP state	Calmodulin	Induction of ERK1/2 by calmodulin inhibition	[81]
	Inhibitory activity on intrinsic GEF-mediated nucleotide exchange	Arabinose-derived bicyclic compound	Mild selective toxicity effect on cells expressing oncogenic RAS-G13D	[82]
	Interfering with RAS-SOS interaction	Synthetic α-helix of SOS1	Downregulation of RAS signaling	[83]
	Blocking the interaction of RAS as a substrate of SOS	DCAI	DCAI blocks the SOS-mediated nucleotide release and inhibits the activation of RAS	[84]
	Inhibition of SOS-catalyzed activation of KRAS	Multiple chemotypes including indoles, phenols, and sulfonamides and their analogues	Blocking binding of KRAS to SOS, and complete inhibition of nucleotide exchange	[85]
	Blocking GDP-GTP exchange	Andrographolide	Reduction in MAPK activation	[86]
	Prevention of GTP loading	SML-10-70-1	Covalent labeling of KRAS, occupation of guanine nucleotide binding site, attenuation of AKT and ERK phosphorylation, and antiproliferative effect on different cell lines	[87]
	Subverting the native nucleotide preference to favour GDP over GTP	6H05 fragment of tethering compounds	Impairing binding to RAF	[76]
	Prevention of GDP exchange by complete inhibition of KRAS-SOS complex	Maleimides	Significant inhibition of the RAS-RAF interaction	[73]
	Blockage of nucleotide association	Alpha helices of SOS1 (SAH-SOS1)	Downregulation of the MAPK signaling cascade	[72]
	Trapping drug-bound KRAS-G12C to its inactive state	ARS-853	Decreased phosphorylation of CRAF, ERK (extracellular signal–regulated kinase), and AKT	[40]
	Disruption of the SOS1-KRAS interaction and thereby stabilization of the inactive GDP-bound conformation of KRAS	Ribonuclease binase	Inhibition of MAPK/ERK signaling	[68]

Continued

Continued

General mechanism of inhibition	Specific mechanism of inhibition	Inhibitor	Result	Reference
	Interfering with GDP release through either inhibition of intrinsic or extrinsic catalyzed exchange mechanisms	ARS-853	Significant loss of KRAS–CRAF interactions, inhibition of MAPK (including pMEK, pERK, and pRSK) and PI3K signaling (pAKT) pathways, loss of Cyclin D1 and Rb expression, an increase in the cell-cycle inhibitor p27, and an increase in hallmarks of apoptosis like PARP (Poly ADP-ribose polymerase) and sub-diploid DNA	67
	Blocking the interaction of K-RAS-G12D with guanine nucleotide exchange factors	KRpep-2d peptide	Induction of large conformational changes in the Switch I and Switch II regions and significant inhibition of RAS activation	74
Disrupting RAS–effector interactions	Inhibition of the interaction between HRAS and RAF1	MCP compounds	Reversion of RAS-transformed phenotype, inhibition of RAS-induced RAF1 activation, and MEK1	88
	Inhibition of RAS-RAF interaction	Sulindac derivative IND12	Restoring epithelial morphology in malignantly transformed MDCK-f3 cells, and inhibition of cell invasion	89
	Inhibition of RAS-CRAF interaction	Non-steroidal anti-inflammatory drug NS398	Inhibition of up-regulation of MAP kinase phosphatases to suppress the ERK-mediated signaling	90
	Inhibition of the interaction of RAS with the RAF-RAS binding domain	MCP compounds	Decreasing active, phosphorylated ERK1/2	91
	Stabilization of a protein conformation that has a weak affinity for effectors	Zn^{2+} cyclen	Inhibition of RAS-RAF interaction	92
	Inhibition of HRAS-GTP and CRAF1 binding	Kobe0065 and its analog Kobe2602	Downregulation of MEK/ERK, AKT, RALA, SOS, and induction of apoptosis	93
	Inhibition the binding of RAS-binding domain of RAF kinases to the RAS	Rigosertib	Disruption of RAF activation, and inhibition of the RAS-RAF-MEK pathway	78

the unsuccessful EGFR targeted therapy for $KRAS^{onc}$-dependent cancers and the difficulty associated with targeting $KRAS^{onc}$ directly, a great deal of effort has been applied to target downstream effector pathways. The specific interaction of RAS family proteins with downstream effectors regulates various cellular functions[3,77,96,97]. Constitutive activation of downstream effector pathways by oncogenic KRAS results in the uncontrolled growth, proliferation, and survival of cancer cells[98]. It is essential to identify the effector pathways that are required for KRAS-driven carcinogenesis to identify pathways that should be targeted for treatment[99].

Two of the best-characterized KRAS effector pathways are the RAF-MEK-ERK and PI3K-AKT-mTOR pathways, which are integral to $KRAS^{onc}$-driven transformation through different signaling cascades[100-102]. These pathways comprise different kinases, providing multiple nodes for potential therapeutic intervention[103,104]. Collectively, studies on targeting the RAF-MEK-ERK and PI3K-AKT-mTOR pathways are divided into two categories. The first series of the studies focused on the identification of compounds

targeting only one of the downstream signaling pathways, including RAF inhibitors, MEK inhibitors, or PI3K inhibitors (**Table 3**).

The results of these studies have shown that, due to the interplay between downstream signaling pathways of KRAS, inhibition of one downstream target leads to the overexpression of its interconnected pathways, creating a drug-resistant phenotype. For example, in response to MEK inhibition, PI3K is activated through a negative MEK-epidermal growth factor receptor-PI3K feedback loop[32,112]. Therefore, novel therapeutic approaches are focusing on the disruption of these multiple nodes, which is only possible through the inhibition of multiple downstream kinases, rather than only one through combination therapy[100-104] (**Table 4**).

According to the valuable results from the combination therapy, extensive studies are moving forward based on multi-targeted therapy for the inhibition of $KRAS^{onc}$ downstream signaling pathways. Recently, a large trial investigated the therapeutic effects of the MEK inhibitor selumetinib and docetaxel in comparison to docetaxel alone, producing results in NSCLC patients with the *KRAS* mutation[114]. Other results from an ongoing trial show a clinical benefit from combination therapy with an investigational MEK inhibitor known as PD-0325901 and palbociclib, an inhibitor of CDK4/6 (PD-0332991), in patients with *KRAS*-mutant NSCLC (NCT03170206) and *KRAS*-mutant PDAC (NCT03454035). In addition, phase II of the other ongoing study on investigational drugs GSK2256098 (focal adhesion kinase inhibitor) and trametinib (MEK inhibitor) was planned to evaluate the antitumor activity of this combination therapy in patients with advanced pancreatic cancer (NCT02428270). BVD-523, an ERK inhibitor, is also currently being tested in combination with nab-paclitaxel plus gemcitabine in a phase Ib trial in patients with metastatic pancreatic cancer (NCT02608229). Another downstream inhibitor is mTOR, a component of the PI3K pathway. The mTOR inhibitor (NCT02329717) PBI-05204 has been tested in patients with stage IV pancreatic cancer. In the other clinical trial, the pan-RAF inhibitor (LXH254) and ERK suppressor (LTT462) are being evaluated as combination therapy for patients with advanced-stage solid tumors with mitogen activated protein kinase (MAPK) alterations, including KRAS-mutant NSCLC (NCT02607813 and NCT02974725). Additionally, phase I/II trials have been initiated to assess the combination therapy of the MEK inhibitor trametinib and the BCL-XL and/or BCL-2 inhibitor navitoclax in patients with *KRAS*-mutant advanced-stage solid tumors (NCT02079740).

Response evaluation criteria in solid tumors

RNA interference (RNAi) is based on a natural process by which RNA molecules inhibit the generation of protein from DNA[115,116]. For example, in the search for novel strategies in the treatment of $KRAS^{onc}$-driven cancer, microRNAs (miRs) have received attention for their role in the regulation of gene

Table 3 Targeting downstream signaling pathways of RAS as therapeutic strategy

Targets	Inhibitor	Results	RAS proteins	Cancers	Reference
RAF kinase	BAY 43-9006	Inhibition of tumor cell proliferation and tumor angiogenesis	KRAS	Colon, pancreatic, and breast cancer	105
MEK	Selumetinib (AZD6244) with Docetaxel	Tumor volume change in mice with KRAS and p53 mutations, but resistance to combination therapy for mice with KRAS and LKB1 mutations	KRAS	Lung cancer	106
MEK	Selumetinib (AZD6244; ARRY-142886)	Pronounced G0/G1 arrest	KRAS and NRAS	NSCLC	107
MEK1/2	Selumetinib with Temozolomide	Enhanced DNA damage and tumor growth inhibition	Unknown	Colorectal cancer	108
MEK	Aelumetinib (AZD6244) with Docetaxel	Improved median overall survival, median progression-free survival, and objective response	KRAS	NSCLC	109
MEK	Selumetinib and Trametinib	Reduction of tumor growth	KRAS	Lung cacer	110
p110α subunit of PI3K	SiRNA and/or BYL719	Reduction of cell viability, induction of apoptosis, and cell cycle arrest	KRAS	Colorectal cancer	111

Table 4 Targeting downstream signaling pathways of RAS as combination therapy

Targets	Inhibitors	Results	RAS protein	Cancers	Reference
MEK and PI3K	NVP-BEZ235 and ARRY-142886	Marked downregulation of PI3K, ERK and downstream signaling	KRAS	Lung cancer	103
MEK and PI3K	PD0325901 and GDC-0941	Enhanced induction of apoptosis, inhibition of cell proliferation, and significant increase in tumor growth inhibition in xenograft models	KRAS	Breast cancer	101
MAPK and PI3K	PI103 and PD0325901	Significant increase of apoptosis after combined treatment	Total RAS	NSCLC	104
MEK and PI3K	GDC-0973 and GDC-0941	Induction of biomarkers associated with apoptosis	KRAS	NSCLC, colorectal, prostate, and pancretic cancer	100
MEK and mTOR	Selumetinib and AZD8055	Xenograft tumor regressions with growth inhibitions, lower phosphorylation of ERK1, S6P, and 4EBP, increasing apoptosis	KRAS	NSCLC and colorectal cancer	102
MEK and AKT	MK-2206 and AZD6244	Improved disease control rate	KRAS	NSCLC	113
Heat-shock-protein 90 (HSP90) and MEK	Trametinib and AUY922	Blocking EGFR/PI3K/AKT activation as well as RAF-MEK-ERK pathway, increasing apoptotic signaling and reduction of tumor growth in xenograft experiments	KRAS	NSCLC	32
MEK1/2 and AKT	Selumetinib (AZD6244; ARRY-142886) and MK-2206	Durable RECIST* tumor shrinkage in NSCLC and low-grade ovarian carcinoma. No clinical responses for colorectal or small-bowel carcinoma	KRAS	NSCLC, ovarian, colorectal, and small-bowel cancer	34

*Response evaluation criteria in solid tumors

expression[30,117]. MiRs are small, single-stranded, highly conserved non-coding RNA molecules that are involved in the control of gene expression[118,119]. These molecules exert their action by binding to target mRNAs to prevent protein production. The degree and nature of the complementarity between the microRNA and target mRNA determines the gene silencing mechanism that will be employed. Perfect complementarity to the mRNA target leads to its subsequent degradation and transcriptional inhibition, while partial complementarity results in the blockade of translation[120]. Therefore, this complementarity plays a key role in regulating the target gene of a particular microRNA. For instance, polymorphisms of the let-7 microRNA binding site in the 3' untranslated region of KRAS leads to an impairment of their complementarity and elevated expression of KRAS[117,121,122].

The dysregulation of microRNAs and their critical roles in carcinogenesis results from the ability of microRNAs to control the expression of oncogenes and tumor suppressor genes[123]. For a microRNA with tumor-suppressor activity, its downregulation promotes tumorigenesis, while overexpression of a microRNAs with oncogenic effects leads to cancer development. Mechanisms responsible for the deregulation of miRs in cancers can be classified as genetic and epigenetic alterations that are observed in cancer cells[124]. Considering KRAS as a proto-oncogene, downregulation of miRNAs that suppress KRAS activation and activation of miRNAs that modulate KRAS expression can lead to cancer development[125]. Some of the microRNAs directly target KRAS, and some of them suppress KRAS activation through other targets (**Table 5**). For example, the results of a study showed that KRAS^{onc} suppresses mir-200 family expression through its downstream effectors JUN and SP-1[119]. An alternative RNA therapeutic approach to miRNAs is through the use of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) from the family of non-coding RNAs[115] (**Table 5**). SiRNAs also regulate gene expression through gene silencing with inhibition of gene translation into protein[126]. Additionally, their similarity in structural characteristics and pharmacokinetic profiles facilitates their use as therapeutics[127].

Regarding RNA-mediated silencing method, there are two strategies to suppress KRAS oncogenic signaling. First strategy is direct in which *KRAS* gene expression is reduced by direct binding of RNAi to KRAS^{onc} mRNA (**Table 5**).

Table 5 Efficacy of RNA therapeutics on KRAS targeting

RNA therapeutics	Direct target of inhibitory RNA	Clinical results	Cancers/Cell lines	Reference
SiRNA	KRAS	Inhibition of growth in metastatic and remetastatic cells as well as in primary tumor cells	Pancreatic cancer	[128]
SiRNA	KRAS	Dramatic reversion of the transformed phenotype, reduction of subcutaneous tumor formation, increase in lag time and noninvasive tumor growth	Colorectal cancer	[129]
MiR-Let-7	c-MYC	Significant growth suppression after treatment with miR-let-7a-1 precursor	Colon cancer	[130]
MiR-143	KRAS	Inhibition of cell proliferation by over expression of micro-143	Colon cancer	[131]
MiR-Let-7g	KRAS	Increase in sensitivity to ionizing radiation after injection of miR-Let-7a	Lung cancer	[132]
MiR-18a*	KRAS	MiR-18a*† repression leads to increased cell proliferation and promoted anchorage-independent growth	Squamous, colon, and hepatic cancer	[133]
MiR-Let-7	RAS and c-MYC	Suppression of proliferation and induction of apoptosis through transfection with miR-Let-7a	Laryngeal cancer	[134]
MiR-Let-7a	KRAS and c-MYC	Significant depression in tumor xenograft weight after injection of miR-Let-7a	Lung cancer	[135]
MiR-Let-7b and MiR-Let-7e	KRAS	Downregulation of miR-Let-7b and miR-Let-7e leads to increased resistance to cetuximab	Colorectal cancer	[136]
MiR-96	KRAS	Transfection with pre-miR-96 results in reduction of cell growth, cell migration, and strong invasive capacity of cells	Pancreatic cancer	[137]
MiR-181a	KRAS	Ectopic expression of miR-181a leads to suppression of cell proliferation and anchorage-independent growth ability	Oral squamous cancer	[138]
MiR-30c	KRAS	Overexpression of miR-30c resulting in inhibition of cell proliferation	Breast cancer	[139]
MiR-Let-7a	KRAS	Chemoradiation therapy resistance after inhibition of miR-let-7a	Colorectal cancer	[140]
MiR-143 and MiR-145	CD44, KLF5, KRAS, and BRAF	Reduction of cell proliferation, migration and chemoresistance by restoring miR-143 and miR-145	Colon cancer	[141]
SiRNA	KRAS	Significant inhibition of proliferation and EMT, and tumor growth and prolonged mouse survival	Pancreatic cancer	[142]
MiR-Let-7g	KRAS and HMGA2	Significant inhibition of cell proliferation, migration, and invasion following overexpression of miR-Let-7g	Hepatocellular cancer	[143]
MiR-193b/365a cluster	KRAS and MAX	Inhibition of cell proliferation, clonogenic potential, and migration with ectopic expression of miR-193b/365a cluster	Cutaneous squamous cancer	[144]
MiR-30b	KRAS, PIK3CD, and BCL2	Suppression of cell proliferation and tumor growth following overexpression of miR-30b	Colorectal cancer	[145]
MiR-96	Ecotropic viral integration site 1 (EVI1) and KRAS	Inhibition of miR-96 leads to attenuation of growth inhibition	Pancreatic ductal cancer	[146]

Continued

Continued

RNA therapeutics	Direct target of inhibitory RNA	Clinical results	Cancers/Cell lines	Reference
SiRNA	KRAS	Decrease in cell number and significant inhibition of tumor growth	Lung cancer	127
SiRNA	KRAS	Decrease in cell viability and proliferation, induction of apoptosis, and attenuation of tumor growth through inhibition of the MAPK pathway	Colorectal cancer	99
MiR-134	KRAS and STAT5B	Inhibition of cell proliferation, induction of apoptosis, cell death, and xenograft tumor growth suppression through overexpression of miR-134	Glioblastoma	147
MiR-1	KRAS and MALTA-1	Inhibition of cell proliferation, increased apoptosis *in vitro*, reduction of tumor growth, and metastasis by overexpression of miR-1	Breast cancer	30
MiR-134	KRAS	Inhibition of proliferation and growth with promotion of apoptosis and sensitivity to the drug following overexpression of miR-134	Gastric cancer	118

[†]Pre-miRNA is further cleaved to generate mature miRNA and antisense miRNA star products (miRNA[*]).

Second approach is indirect, which is based on the inhibition of synthetic lethal interactions. Synthetic lethality is a phenomenon through which a genetic alteration leads to cell death only in the presence of another genetic perturbation. Mechanistically, synthetic lethal interactions can involve genes that are functionally connected[148]. In cancer cells, aside from pathways directly controlled by oncogenes, there are several non-oncogene-targeted pathways, which are involved in the process of transformation[149]. Thus, oncogenes require additional support from other genes to maintain the oncogenic state[26]. A major challenge in cancer treatment is the identification of targets that can be inhibited for the selective killing of cancer cells while sparing normal cells. Synthetic lethal interactions between oncogenes and non-oncogenes in cancer cells increase the sensitivity of cancer cells to selective therapeutics in comparison to normal cells[150].

The *KRAS* mutation predisposes cancer cells to additional dependencies on the activity of genes that are not directly regulated by KRAS. This phenomenon can provide an approach for the selective treatment of KRAS^{onc}-driven cancers according to the synthetic lethal interactions[26,151]. The KRAS signaling pathway is complex, so several potential synthetic lethal targets are required for the initiation or maintenance of *KRAS* mutant tumors. To identify critical nodes in the signaling pathways regulating aberrant KRAS^{onc} signaling, RNA silencing technologies could be exploited[152]. These newly identified synthetic lethal interactions lead to novel therapeutic opportunities. Additionally, small-molecule synthetic lethality screens have resulted in the identification of the selective effect against *KRAS* mutant cells compared with wild-type cells[31,153]. It should be noted that inhibition of synthetic lethal interactions is not only accomplished by RNAi, but also with small molecules. A summary of researches on the screening of synthetic lethal interactions with KRAS^{onc} through RNA silencing methods or small molecules is provided in **Table 6**.

Antisense oligonucleotides directed against KRAS^{onc} have indicated a therapeutic benefit in laboratory studies, opens up multiple effective possibilities for suppressing KRAS activity, and preventing the feedback response and drug resistance while facilitating combination therapy[99]. Despite the tremendous potential of RNA-based therapies, the successful application of this technology is currently limited. RNAs are inherently unstable, and therefore there is lack of efficient delivery of sufficient amounts to the target tissue. Additionally, toxicity due to off-target effects and the induction of immune system responses also represent difficulties related to this approach[115,158,159].

Targeting the immune system

Cancer immunotherapy for patients carrying the *KRAS* mutation has become a clinical oncology reality. *KRAS*-G12D knockdown cells show increased production of interleukin 18 by the host immune system, leading to a dramatic reversion of the transformed phenotype and reduction of the proliferation rate of cancer cells[129]. Increases in NK cells and

Table 6 Inhibition of synthetic lethal interactions of KRAS as a therapeutic strategy

Inhibitor	Cancer cell	Target	Conclusion	Reference
Oligonucleotide-directed mutagenesis	NIH 3T3 fibroblasts	RAC/RHO pathway	Impairment of RAS-mediated transformation	154
SiRNA	Human lung cells	PKC	Apoptosis induction and suppression of the growth of KRAS mutant human lung tumor xenografts	155
ShRNA	Human lung epithelial cells	TBK1	*In vitro*: reduction of cell viability. *In vivo*: inhibition of growth of tumor xenografts and induction of apoptosis	152
ShRNA	Colorectal cancer cell lines	THOC1	Reduction of mutant cell fitness percentage	26
ShRNA	Colorectal cancer cell line	COPS4	Impaired growth on adherent surfaces	26
BI-2536 and shRNA	Colorectal cancer cell lines	PLK1	Increased toxicity towards RAS mutant cells and reduction of cell fitness percentage	26
MiR-Let-7	Colon cancerc-MYC	c-MYC	Significant growth suppression after treatment with miR-let-7a-1 precursor	130
ShRNA, MG132, and Bortezomib (Velcade)	Colorectal cancer cell line	Anaphase promoting complex (APC) subunits	Reduction of mutant cell fitness percentage and G2/M arrest	26
ShRNA	Murine embryonic fibroblasts	ATR-CHK1 pathway	Suppression of proliferation due to the synergistic increases in genomic instability	31
ShRNA	Human NSCLC cell lines	Wilms tumor 1 (WT1)	Induction of senescence and decrease of proliferation	156
ShRNA and siRNA	Colon cancer	Snail2	Impaired colony formation in soft agar and suppressing the malignant phenotype by reversion of EMT	157
SiRNA and Bortezomib	Human colon cancer cell line: HCT-116	CDC6 and proteasome	Induction of apoptosis	149
MG-132 and proteasome inhibitor I	Human colon cancer cell line: HCT-116	Proteasome	Pro-apoptotic and loss of viability responses	149
Bortezomib, Topotecan, and Doxorubicin	Human colon cancer cell line: HCT-116	Proteasome and topoisomerase	G2/M arrest	149
SiRNA and Bortezomib with Fasudil	NSCLC cell lines	Proteasome components, IL-1 signaling, and Rho-signaling pathways. regulated by GATA2	Reduction of mutant cells viability, tumor burden, tumor number, and average tumor size	150
MiR-200 family	Lung and breast cancer	BCL2	Restoration of mir-200 resulting compromised KRAS-induced cellular transformation, apoptosis, EMT transition, and tumor formation	119
ABT-263 and Selumetinib	Colorectal, lung, and pancreatic cancer	BCL-XL and MEK	Promotion of apoptosis	151
Navitoclax, G-963, and GDC-0941	NSCLC and pancreatic cancer	BCL2/BCL-XL, MEK, and PI3K	Suppression of AKT activation resulting in increased cytotoxicity, cell population with sub-2N DNA content, and PARP‡ cleavage	153

‡ Poly (ADP-ribose) polymerase

antibody-dependent cell-mediated toxicity after combination therapy with lenalidomide and cetuximab lead to increases in circulating naïve and central memory T cells in patients with KRAS-mutant colorectal cancer[160]. The *KRAS* mutation induces increased expression of programmed cell death 1 ligand 1 (PD-L1)[161]. According to these findings, Ebert et al.

showed that anti-PD-L1 antibodies significantly reduced tumor size in MEK inhibitor-treated mice with the *KRAS* mutation. Thus, it seems that the combination of immunotherapy and anti-proliferative agents, such as MEK inhibitors, provides higher anti-tumor activity[162]. Genetic alterations are specific to cancer cells and are not present in normal cells; thus, treatments that specifically target the protein product of these genetic aberrations may provide a clinical benefit in the absence of normal cell toxicities. Although mutant KRAS proteins themselves are not strongly immunogenic, efforts are underway to enhance the ability of the immune system to recognize KRAS mutant peptides as neo-epitopes. For example, specific immunogenic mutations could help to recognize KRAS mutant variant peptides of the most frequent *KRAS* mutations, such as G12V and G12D, by specific T cell receptors[163]. In this way, to develop more effective personalized immuno-therapy for patients with the *KRAS* mutation, Rosenberg's team isolated tumor-infiltrating lymphocytes (TILs) with the ability to specifically target the *KRAS* mutation. The findings of that study, which were presented in December 2016, introduced, for the first time, a novel immunotherapy-based strategy, called adoptive T cell transfer immunotherapy. These results validated the possibility of using personalized T cell receptor gene therapy against multiple types of cancer expressing this common mutation or other types of *KRAS* mutations[163].

Thus, the purpose of recent studies has been the identification of immune-editing of T cells during tumor development, as well as the determination of their potential applications for tumor-specific immunotherapy[164].

According to the brilliant results from immunotherapy, treatments focused on altering the immune system for patients suffering from KRAS^{onc}-driven cancers have been intensively investigated in recent years, with new achievements. In one study, the efficacy of immune checkpoint inhibitors among NSCLCL patients was found to correlate with the *KRAS* mutation as a molecular smoking signature[165]. Other evidence indicates that the co-mutation of *TP53* and *KRAS* in lung adenocarcinoma can be exploited as a potential predictive marker for effective immune checkpoint blockade immunotherapy[164]. Clinical trials have also been initiated for the *KRAS*-G12D-specific cancer vaccine TG01/ GM-CSF either alone or combined with gemcitabine. The initial results of these trials have shown an induction of the immune responses in response to TG01/GM-CSF plus gemcitabine combination therapy[166]. A study to evaluate the efficacy and safety of cobimetinib plus atezolizumab and atezolizumab monotherapy versus regorafenib in participants with metastatic colorectal adenocarcinoma is currently ongoing as a phase III trial (NCT02788279). The initial findings suggest that this therapeutic strategy is helpful in improving the immune response. One trial examining the combination therapy of a newer CDK4/6 inhibitor, abemaciclib, with the immune checkpoint inhibitor pembrolizumab is currently ongoing in NSCLC patients with the *KRAS* mutation[167]. New achievements have been observed in these studies against human cancers (**Table 7**), represent the need for further studies to enhance immunotherapeutic efficacy in some patients.

Other approaches

Despite important strides made in the development of targeted therapy for KRAS^{onc}-mediated cancers, no therapeutic approaches are clinically available. In recent years, a deeper understanding of the critical parameters involved in the promotion of KRAS^{onc}-driven tumorigenesis has been considered for the development of new therapeutic options. In this part of the article, we review these new achievements and discuss multiple lines of evidence of novel key pathways that are recognized to interact with other previously identified KRAS-regulated survival pathways to transduce signals of carcinogenesis. The data suggest that co-targeting of these newly and previously recognized KRAS^{onc}-regulated pathways has significant clinical potential.

Inhibition of stem cell program

Cancer stem cells (CSCs) are defined as tumor-initiating cells with self-renewal capacity. They are considered to be responsible for cancer initiation, progression, metastasis, drug resistance, and treatment relapse[168]. The *KRAS* mutation has been shown to preferentially alter the profile of gene expression to induce embryonic stem cell-like features[169]. For example, the expression of some genes is known to be upregulated in the presence of the *KRAS* mutation, including fibroblast growth factor receptor 1 (FGFR1), which plays a common role in both embryonic and cancer development, LCK, the transcriptional silencing of which is required for embryonic stem cell differentiation, and the induced-pluripotency factor SOX2, which reprograms differentiated cells to pluripotency. In contrast, KLF4 expression was suppressed in KRAS mutant colon cancer cells, which is consistent with its induction of multiple cell lineage differentiation in the intestine[18]. Additionally, a KRAS-centric mechanism would apply in the context of epidermal-mesenchymal transition (EMT) to generate CSCs

Table 7 Studies on immune system targeting RAS-driven cancers

Immunomodulator	Mechanism of action	Results	Cell line	Reference
Host immune system	$KRAS^{GD12}$- knockdown cells increased production of interleukin 18 by host immune system	Dramatic reversion of the transformed phenotype, reduction of proliferation rate subcutaneous tumor formation	$KRAS^{GD12}$ murine C26 colorectal cancer cells	129
Lenalidomide in combination with cetuximab	Increase in NK cells and antibody dependent cell-mediated toxicity	Increases in circulating naïve and central memory T cells	KRAS-mutant metastatic colorectal cancer cells	160
Engineered T cells	Activity of T-cell receptors of engineered T cells against the HLA-A*11:01+ tumor lines presenting mutated KRAS variants	Reduction of tumor growth in xenograft model	KRAS mutant human pancreatic tumor lines	163
MEK inhibition in combination with anti-PD-L1	Induction of the accumulation of antigen-specific CD^{8+} T cell effectors in tumors and prevention of the "exhaustive" T cell death	Durable tumor regression	CT26 colon carcinoma cell line harboring mutant $KRAS^{G12D}$	162
Pembrolizumab	PD-1 blockade immunotherapy	Remarkable clinical benefit to PD-1 inhibitors	Lung adenocarcinoma	164

through the WNT pathway[170].

Other results have indicated that oncogenic KRAS activation in the genetic background of loss-of-function of adenomatous polyposis coli (APC) results in enhanced CSC activation by increasing both intracellular stabilization of β-catenin and the MAPK pathway[171,172]. Furthermore, endodermal progenitors expressing *KRAS*-G12V do not differentiate upon retinoic acid treatment and continue to proliferate and maintain stem cell characteristics[173]. Several studies have described the *KRAS* mutation as a driver of stem cell-like properties of cancer cells. Thus, inhibition of multiple key pathways involved in embryonic stem cell signaling represents a novel therapeutic strategy. Le Rolle et al.[18] showed that inhibition of KRAS mutant colon tumors with miR145, an epigenetic regulator and an embryonic stem cell inhibitor, suppressed their malignant growth. Data suggest that salinomycin, the most potent cancer stem cell inhibitor with potential efficacy in human cancers, specifically disrupts $KRAS^{onc}$ nanoscale membrane organization, effectively reducing effector recruitment to $KRAS^{onc}$, which then compromised at least MAPK signaling and proliferation[170]. Ophiobolin A, another candidate CSC drug, has been found to possess higher potency than salinomycin and exert its KRAS4B-specific activity through the inactivation of calmodulin[170].

Based on the role of the $KRAS^{onc}$ in stemness, α-Mangostin-encapsulated PLGA [poly (D, L-lactic-co-glycolic acid)] nanoparticles show inhibitory effects on carcinogenesis in transgenic mice carrying the *KRAS* mutant allele through the downregulation of pluripotency maintenance factors (c-MYC, NANOG and OCT4) and stem cell markers (CD24 and CD133)[174]. Overall, these data suggest that targeting multiple signaling pathways of cancer stem cell activation induced by the KRAS mutation could be an attractive therapeutic approach.

Targeting receptor tyrosine kinases (RTKs)

A growing body of evidence suggests that the *KRAS* mutation may serve as a predictive resistance marker to guide the use of anti-EGFR therapy. Multiple studies have demonstrated that patients with mutations in *KRAS* do not appear to experience a clinical benefit from anti-EGFR monoclonal antibody treatment[175]. In cancers with *KRAS* mutations, part of the cell survival and proliferation pathways could still be due to the activation of upstream RTKs other than EGFRs. Therefore, another possible approach to target tumors with *KRAS* mutations is through the inhibition of such critical RTKs that contribute to the enhanced prosurvival. The type 1 insulin-like growth factor receptor is a promising target in different types of cancers, including colon cancer[176]. The PI3K signaling pathway is a common downstream effector of both IGF-1R and KRAS. Thus, blockade of IGF-1R using different monoclonal antibodies or tyrosine kinase inhibitors is theoretically relevant for the treatment of patients with $KRAS^{onc}$-driven cancers[8]. Although patients with the *KRAS* mutation show resistance to EGFR-targeted therapy, preclinical data have indicated that combination therapy with

IGF-1R and EGFR kinase inhibitors results in synergistic growth inhibition in colorectal cancer cell lines[9]. Hurwitz et al.[10] showed a clinical benefit following the treatment of patients with bevacizumab as an anti- vascular endothelial growth factor (VEGF) therapy. Data have also shown that, unlike anti-EGFR therapy, anti-VEGF therapy functions independently of the *KRAS* mutation status, revealing even greater clinical significance.

Stabilization of the G-quadraplex

G-quadruplexes (G4) are special secondary structures containing runs of guanines separated by other bases[177]. The localization of G4 in the human genome was found to be non-random, indicating their important role in the regulation of functional regions. Significantly, G4 are more frequent in oncogenes or regulatory genes than in house-keeping or tumor suppressor genes. Their higher distribution in the promotors of oncogenes suggests a possible involvement of G4 in cancer[178]. Genome-wide analysis of human cells has revealed the role of these structures is gene-silencing through the inhibition of replication, transcription, and translation[35]. Therefore, the stabilization of guanine-rich regions located in the oncogene promoters represents a highly valuable new molecular target for the development of novel anti-cancer therapeutics[177]. It is now evident that the core promoter region of KRAS contains silencing G4 elements[179]. G-to-T knockout mutations in the G4-forming regions of the *KRAS* promoter were found to disrupt or abrogate G4 formation. In addition, stabilization of the *KRAS* promoter by the cationic porphyrin TMPyP4 leads to a significant decrease in *KRAS* expression[180]. The interaction of G4 of the *KRAS* promoter with natural polyphenols, such as ellagic acid and curcumin, has also been confirmed by UV-vis spectroscopy. Significantly, the melting temperature of the G-quadruplex is increased, indicating its stabilization upon interaction with polyphenol ligands[35].

Inhibition of inflammation

KRAS-driven tumorigenesis is tightly connected with tumor-promoting inflammation, which increasingly represents another promising therapeutic strategy[181]. According to recent clinical data indicating the role of inflammation in the carcinogenesis related to the *KRAS* mutation, targeting inflammatory signaling pathways seems to be an essential component of therapy for tumors with *KRAS* mutations[182]. Different cellular pathways, which are modulated by KRAS and induce inflammation, include JAK/STAT, NF-κB, MAPK, and immune checkpoint signaling pathways[36]. For example, the *KRAS* mutation contributes persistent pancreatitis induced by cerulein. In this situation, suppression of inflammation by deletion of IKK-β and inhibition of NF-κB activity interferes with dysplasia. In contrast, overexpression of IKK-β cooperates with the KRAS mutant allele to promote oncogenesis[183].

A different study indicated that while persistent KRAS activation drives the secretion of STAT3 pathway mediators, activation of STAT3 results in the amplification of KRAS^{onc} carcinogenesis through the upregulation of anti-apoptotic and pro-proliferative proteins[184]. Co-administration of azoxymethane (AOM) and dextran sodium sulfate (DSS), respectively, as carcinogenic and inflammatory agents, results in a significant decrease in the latency of KRAS^{onc}-driven tumor formation[185]. Given the presence of inflammatory stimuli in a *KRAS* mutation background as positive feedback promoting KRAS^{onc}-associated carcinogenesis, targeting each of the mentioned signaling pathways would likely lead to the development of a mechanism for disease control.

Targeting metabolic pathways

Metabolic reprogramming of cancer cells due to oncogenic mutations is critical for cell growth and survival. Data show that the KRAS oncoprotein confers metabolic robustness for the acquisition of cellular metabolism networks to convert carbon sources into biomass[186]. The metabolic features of KRAS^{onc}-driven cancers can be explained through the reprogramming of glucose, amino acids, and lipid metabolisms[37]. Cancer cells harboring KRAS^{onc} promote the glycolytic switch, glucose uptake, increased channeling of glucose-derived metabolites into the tricarboxylic acid cycle, and activation of glucose-dependent biosynthetic pathways[187]. For example, it has been reported that the *KRAS* mutation increases the expression of glucose transporter-1 (GLUT1) and several rate-limiting glycolytic enzymes[188]. Interestingly, the induction of metabolic changes is dependent on the content of the *KRAS* mutant allele of cancer cells. Thus, glycolytic gene expression was markedly enhanced in *KRAS*-G12D/G12D relative to heterozygous lung tumor cells[187]. One mechanism by which KRAS^{onc} aberrantly regulates metabolic networks is through the reprogramming lipid metabolism by the promotion of cellular uptake, retention, accumulation, synthesis, and oxidation of fatty acids. For instance, lung cancer cells carrying the *KRAS* mutation are highly dependent on the activity of acyl-coenzyme A synthetase long-chain family member 3 (ACSL3)[28,189]. Mutated *KRAS* promotes

lipogenesis through the induction of fatty acid synthase, leading to lipid signatures of human lung cancer cell lines[189]. Other results have shown that the RAS mutation leads to the reprogramming of *de novo* lipogenesis of cancer cells by scavenging serum fatty acids[190]. Emerging evidence from different research groups indicates that *KRAS* mutations are associated with changes in amino acid metabolism[191]. Reprogramming of glutamine metabolism in KRAS^{onc}-driven cancers is the most important alteration in amino acid metabolism. While most cells utilize glutamate dehydrogenase 1 for conversion of glutamate into α-ketoglutarate, cancer cells carrying the *KRAS* mutation convert glutamate to aspartate[191]. The increased requirement for branched-chain amino acids (BCAAs) is a very early phenomenon during tumor development, similar to some types of KRAS^{onc}-driven cancers[192]. As mitochondrial activity is required for metabolic changes in cancer cells, autophagy as a mechanism for the elimination of defective mitochondria is crucial for tumor growth. Loss of essential autophagy genes in KRAS^{onc}-driven cancer impairs effective mitochondrial function and suppresses tumor progression, emphasizing the role of autophagy in the intracellular nutrient supply[193]. These reports indicate that the *KRAS* mutation creates unique metabolic dependencies that could be exploited for anti-cancer therapy.

Targeted RNA replacement

Tetra hymena group I intron-based trans-splicing ribozyme is specific therapeutic tool with ability to discriminate the target RNA resulting in specific and high-fidelity cleavage reaction of its target[194]. Moreover, ribozymes can specifically transfer the therapeutic gene into cancer cells expressing target RNA. This specific trans-splicing reaction with the ability of discrimination target RNA from non-target one, even with a single nucleotide difference, makes it as an attractive novel treatment strategy for *KRAS* point mutations. Regarding *KRAS*-G12V mutation as one of the most prevalent point mutation, Tetra hymena group I intron-based trans-splicing ribozyme designed for selective cleavage of *KRAS*-G12V transcript[195]. An accurate and specific intracellular trans-splicing reaction of the designed ribozyme systems with the *KRAS*-G12V target RNA, leads to efficient reduction of transcript level. Except that replacement of RNA, concurrent induction of suicide gene activity resulting in cytotoxicity and effective retardation of cancer cells harboring *KRAS* mutation[196]. Moreover, trans-splicing and therapeutic anti-cancer gene activity was selectively and efficiently induced only in *KRAS*-mutant cancer cells without targeting of cells expressing wild-type KRAS[195].

Oncogene-induced senescence

Oncogene-induced cellular senescence (OIS) is a complex mechanism of tumor suppression which is thought to be triggered by aberrant activation of oncogenic signaling[197]. Undisputed role of RAS^{onc} in different human cancers, necessitate studies on the RAS^{onc}-induced senescence as an alternative treatment strategy. Senescence is not a simple mechanism triggered by only linear series of events and multiple components are required to establish a senescence response. Accordingly, detailed molecular mechanisms underlying OIS should be completely understood to provide adequate mechanistic insight for implementation of RAS aberrant oncogenic signaling against themselves as a potential anti-cancer strategy[198].

Basically, there are three pathways which are recruited by KRAS^{onc} to induce senescence which are also interconnected. The first pathway is transcriptional repression of pro-proliferative genes like E2F target genes. In addition to the transcriptional repression, a second pathway that is believed to mediate KRAS^{onc}-induced senescence is the DNA damage pathway. Oncogene activation induces aberrant DNA replication events, leading to replication stress and subsequent DNA damage[198]. Consequently, DNA damage and accumulation of proteins involved in DNA damage response, like ATM and CHK2 results in senescence induced by oncogene activation. Finally, a third pathway, which is essential for senescence and recruited under RAS activation is senescence-associated secretory phenotype (SASP). Studies have recognized that SASP mediates RAS^{onc}-induced senescence, through the secretion of specific proteins like C/EBPβ transcription factor[199]. Notably, the neurofibromatosis type 1 (NF1), encoding a RAS-specific GAP, has been implicated in OIS[200]. In this context, suppression of Ras and/or PI3K are sufficient to induce senescence, and these events on their own can activate the known downstream mediators of the senescence response (Rb and p53) through a variety of mechanisms[200] (**Figure 1**). Moreover, in BRAF-driven melanomagenesis, loss of *NF1* cooperates with *RAF* mutations by increasing PI3K/AKT signaling and preventing entry into OIS[201,202]. While the significant role of the oncogenic RAS in human cancers has been proved for many years, a better understanding of the molecular basis of RAS^{onc}-mediated senescence, allows the delineation of new therapeutic approaches surprisingly aimed at engagement of oncogenic signaling against oncogenic signaling.

Conclusions

More than 30 years of intensive research and tens of thousands of published studies have provided valuable insights into the biology, biochemistry and biophysics of RAS family proteins. Signal transduction of RAS (most notably KRAS) is regulated by three classes of canonical interacting partners, including regulators that control activation of the GTPase cycle (by GEFs), its inactivation (by GAPs), and a wide spectrum of effectors (e.g., RAF kinase and PI3 kinase) that initiate signaling cascades downstream of RAS and RAS-like proteins. We have gained deep knowledge about their membrane trafficking, structure-function relationship, mechanisms of GDP/GTP binding and accelerated nucleotide exchange by GEFs, intrinsic and GAP-stimulated GTP hydrolysis, interaction with effectors and activation of diverse signaling pathways. However, these studies have their own eligibility confinement: cell-free investigations have been predominantly carried out in the absence of lipid membrane, using defined domains rather than full-length proteins, and cell-based studies have mostly been performed *via* the heterologous expression of tagged genes and their variants in methodologically congenial cell lines. As the omics era is coming to an end and research has decelerated, many new movements have emerged, especially due to the accessibility of new technologies. Several novel mechanisms have been uncovered that have extended our understanding of the role of protein-protein/protein-lipid interactions and various types of post-translational modifications in the modulation of RAS protein activity. Another issue is the activation mechanism of regulators and effectors. Notably, the identification of additional components of the RAS interaction networks is a critical step towards understanding both the relationship between RAS proteins and the selective activation of respective effectors, as well as the molecular signatures required for the spatiotemporal integration and activation of GEFs and GAPs. The identification and functional reconstitution of specific interaction networks by using appropriate liposomes and full-length effector proteins may eventually provide fundamental insights into the functional characterization of multiprotein complexes of RAS and the complete identification of regulatory mechanisms. In this context, an interesting issue, which is increasingly appreciated, is a RAS-membrane interaction that appears to generate RAS isoform specificity with respect to regulator and effector interactions. Currently, it has become more evident that an increasing number of additional RAS binding partners are critical in modulating and integrating RAS in various signaling networks at biological membranes. This phenomenon is likely achieved by scaffold proteins, including CAM, GAL1, GAL3, IQGAP1, NCL, NPM1, SHOC2, SPRY, SPRED1 and GAB1, which may modulate isoform specificity at specific sites of the cell. However, the roles of these additional RAS interaction proteins as novel modulators of RAS signaling remain unclear. Hence, elucidation of the RAS signal transduction requires not only RAS-effector interactions but also additional structures and the interplay of multi-protein complexes. Keeping this in mind, accumulating evidence supports a role for cell type-dependent RAS paralog functions that should prompt future efforts to examine the respective pathways in a more context-specific manner. Excluding driver mutations, passenger mutations accumulate and frequently escape natural negative selection, resulting in several oncological outcomes[203]. In parallel with standard tumor profiling methods, high-throughput technologies, such as next-generation sequencing, have been employed to shift the treatment paradigms. Thus, further characterization of the heterogeneous identity of patient tumor tissue exploring all specific molecular aberrations along with the specific *KRAS* mutation, seems to be critical for an effective therapy[204,205]. Such efforts could lead to the identification of disease-specific therapeutic opportunities. The other novel technology is phosphoprotein analysis through kinome profiling, which provides evidence of signaling pathways that are activated in a patient's tumor[206].

The authors of this review article conclude that translating our knowledge of different treatment frameworks to the clinic *via* targeted therapy of the KRAS^{onc} and personalized immune-therapy may be the best strategies to dramatically improve patient outcomes. In summary, we are at the beginning of a new series of attempts to treat KRAS^{onc}-driven cancers by directly targeting the protein or through personalized targeted therapy with high-throughput or immunotherapy-based strategies. This new wave of personalized studies provide hope for thousands of patients suffering from KRAS^{onc}-driven cancers.

Acknowledgments

Authors are thankful to Dr. Seyed Ali Jafari (Mashhad University of Medical Sciences) for insightful comments, and to Dr. Saeideh Nakhaei-Rad for her valuable suggestions. We thank American Journal Experts and Ms. Diana Inanlou for language edition of the manuscript. M.R.A. was supported by the European Network on Noonan Syndrome and Related Disorders (NSEuroNet, Grant No. 01GM1602B), and the German Federal Ministry of Education and Research

(BMBF): German Network of RASopathy Research (GeNeRARe, Grant No. 01GM1519D & 01GM1902C).

Conflict of interest statement

No potential conflicts of interest are disclosed.

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Cite this article as: Saliani M, Jalal R, Ahmadian MR. From basic researches to new achievements in therapeutic strategies of KRAS-driven cancers. Cancer Biol Med. 2019; 16: 435-61. doi: 10.20892/j.issn.2095-3941.2018.0530