Analysis of the KRAS gene: Implications for cancer and targeted therapies.

Maider Uriarte

1.Introduction

The impact of cancer on society is of great interest to the scientific community as it is one of the most deadly illnesses. Major advances in cancer therapy have improved patient survival and reduced morbidity. Luckily, increasing research in oncogenes and tumor suppressor genes have increased our understanding of cancer. Recently, it has been discovered that 90% of all human cancers involve point mutations and abnormal activation of RAS proteins. The RAS proto-oncogene, when mutated, is unable to transform normal cells into tumor cells.

2.Overview of KRAS function in healthy cells

In healthy cells, the Kristen RAS oncogene, KRAS, mainly acts as a binary on/off switch for many cellular processes. Its most well-understood role is in the transmission of extracellular signals that lead to cell proliferation or differentiation (Cox et al., 2014; Downward, 2003). Normally, cells will only divide in response to growth-promoting signals. When growth signals are absent, cells will exit the cell cycle and enter a dormant "G0" state. If a cell receives proliferative signals during G0, KRAS and other signaling pathways can stimulate gene expression that initiates cell growth and division (Pylayeva-Gupta et al., 2011). Continual signaling can cause cells to bypass the G0 phase and divide uncontrollably. This is the case in many cancers, and the frequency of KRAS activation in various cancers supports a role for KRAS in maintaining that proliferative state (Stephen et al., 2014; Vogelstein et al., 2013). An important point to make is the difference between transient KRAS activation by a growth signal and continual activation as a result of mutations that can lead to the uncontrolled proliferation of cancer cells. The latter is what gives mutated KRAS its oncogenic properties (Malumbres & Barbacid, 2003).

Differentiation is another process that KRAS regulates. In hematopoietic cells, for example, transient KRAS activation is known to stimulate the production of macrophages and granulocytes (Simanshu et al., 2017). Constitutive activation, on the other hand, blocks the differentiation of precursor cells into mature blood cells. This continual activation of KRAS due to mutations is a harmful process. It leads to uncontrolled cell proliferation and blocks the normal differentiation of cells, contributing to the development and progression of cancers (Prior et al., 2020).

2.1 Regulation of cell growth, proliferation, and differentiation

KRAS has also been shown to inhibit differentiation of various cell types. For example, KRAS activity inhibits differentiation of hematopoietic cells into erythrocytes and the differentiation of pre-adipocyte cells to adipocytes (Stephen et al., 2014). These inhibitory effects are primarily due to KRAS activity preventing differentiation resulting in decreased cell growth rate and an entry into a quiescent state that would occur upon cell differentiation (Downward, 2003).

KRAS has been shown to have marked effects on stimulating the production of ribonucleic acid (RNA) and ribosomal RNA (rRNA) in the nucleolus. This action increases RNA translation and ultimately results in increased production of proteins involved in cell growth and proliferation as well as those necessary for the synthesis of DNA and cell cycle progression. The result is an increase in cell growth and proliferation and ultimately an increase in cell mass (Cox et al., 2014).

In order to understand how KRAS functions in healthy cells, it is important to understand the effects of KRAS on normal cellular functions. KRAS has effects on cell growth, proliferation, and differentiation by way of interacting with other molecules to transmit growth-promoting signals. One of the most thoroughly researched areas of KRAS function involves its efforts to promote cell cycle progression from the G1 to the S phase (Malumbres & Barbacid, 2003).

2.2 Transmission of extracellular signals to the nucleus

When receptor proteins of a stimulated cell are activated by growth factors such as Epidermal Growth Factor (EGF), they in turn activate the GDP/GTP exchange activity of RAS by binding the protein guanine nucleotide exchange factor (GEF), which catalyzes the transition of the RAS bound GDP to GTP (Bos, 1989; Vetter & Wittinghofer, 2001). Once GTP is bound, RAS is in its active state and can interact with myriad of effectors (for example Raf kinases, RalGDS, PI3 kinase), many of which activate different signaling cascades, though all contribute to creating a biological effect in the cell (McCormick, 1994; Rodriguez-Viciana et al., 1994). By fast and transient binding, GTP-bound RAS activates a protein kinase, also known as MAPK, which catalyzes the phosphorylation of transcription factors that result in the increased production of certain proteins, for example increased growth or division (Widmann et al., 1999). This up-regulation of transcription factor phosphorylation/expression can also be achieved through an increase of MAPK activity and the down-regulation of MAPK phosphatase activity, both of which are processes that RAS is also implicated in through various mechanisms. An increase in p190 RhoGAP phosphorylation and the subsequent decrease in RhoA activity is another effect of RAS activation (Tapon & Hall, 1997). This process leads to the disassembly of actin stress fibers and the increase of integrin-mediated cell migration, though the importance of this pathway in RAS signaling is disputed when compared to the MAPK pathway. A third mechanism involves the activation of the PI3 kinase pathway, which results in increased phosphorylation of

Akt and the subsequent inactivation of GSK-3, all resulting in a net increase in glycogen synthesis and gene expression (Cantley, 2002). These various pathways are highly effective and are the reason that only a small increase in activated RAS is needed to result in a fast change in cell physiology. The rate of these RAS-catalyzed reactions can be altered by GAP and effector phosphorylation, thus giving a variable timing signal to different effectors and the diverse range of biological effects in the cell.

2.3 Participation in normal cellular processes such as metabolism and cytoskeletal dynamics

KRAS is involved in nucleotide production, which is vital for cellular replication and may account for the increased rates of both in neoplastic cells. Owing to the limiting factors in glycolysis and subsequent metabolic processes, it is likely that cancer cells with constitutively active KRAS have overactivation of those specific metabolic pathways. It may be possible to develop targeted treatments with low volume/short-term dosing chemotherapeutic agents in order to deprive the cancer cells of necessary metabolites (DeBerardinis & Chandel, 2016; Ying et al., 2012).

Metabolism has long been linked to cancer, with Otto Warburg first describing tumor cells as having high glycolytic rates in the presence of oxygen (Warburg, 1956). Metabolism of cancer cells has since been shown to be different from normal cells, with mutations in oncogenes and tumor suppressor genes allowing the uptake and storage of energy in glucose to bypass metabolic pathways in order to generate glycolytic intermediates and decrease hydrogen ion production, an adaptation for the acidic microenvironments often found in solid tumors.

Normal cellular processes include metabolism and cytoskeletal dynamics, which are both important for cell maintenance. Metabolism of a cell allows the generation of energy and by-products necessary for all other cellular functions. Cytoskeletal dynamics enable the cell to change its shape and also play an important role in motility.

3. Mutations in KRAS and Cancer

It is common for a variety of tumors to express more than one type of KRAS mutation. The mutations can also differ among the subtypes of tumors and progression of one disease to another (Prior et al., 2012).

- G12D: This leads to an accumulation of KRAS-GTP in the cell. It has been shown that this mutation results in the downregulation of downstream pathways such as Raf-MEK-MAPK (Cox & Der, 2010). This is due to the G12D mutation interfering with the binding of GTP-KRAS and Raf. The result is that GTP-KRAS is unable to bind to the downstream effector and thus inhibits the activation of the pathway (Pylayeva-Gupta et al., 2011).

- G12C: This mutation is similar to the G12D mutation; however, it results in a different biochemical change. G12C has been linked with an increase in microtubule affinity. This results in the rapid transport of GDP-KRAS to the plasma membrane where GDP-KRAS is converted to GTP-KRAS. Similar to G12D, there is an accumulation of GTP-KRAS in the cell and inhibition of the Raf-MEK-MAPK pathway (Moore et al., 2020).

KRAS is a small GTPase that acts as a molecular on/off switch and plays a key role in transmitting signals from outside the cell to the nucleus via the MAPK pathway (Prior et al., 2012). KRAS is frequently mutated in cancer, leading to inactivation of its GTPase activity. This leads to the constitutive activation of the protein and results in continuous signaling. KRAS mutations occur in 90% of pancreatic cancers, 22% of colon cancers, and 17% of lung cancers (Almoguera et al., 1988). Studies have shown that mutations in codon 12 of the KRAS gene result in a protein with reduced GTPase activity and an increased affinity for GTP. This results in a failure to switch off the activated state of KRAS (Cox & Der, 2010).

3.1 Prevalence of mutations in different cancer types

Mutations in the RAS gene are common in human cancer - occurring in about 30% of all human tumors - and are especially prevalent in certain cancer types (Prior et al., 2012). The three main RAS genes (HRAS, KRAS, and NRAS) are mutated in a variety of tumors. Data from The Cancer Genome Atlas suggests that mutations are often mutually exclusive, meaning that one gene is mutated in isolation (Cancer Genome Atlas Research Network, 2012). For example, pancreatic cancer has KRAS mutation in over 90% of occurrences, while it is much lower in thyroid cancer (Almoguera et al., 1988). Diverse genomic sequencing efforts in recent years have allowed for a clearer understanding of the prevalence of RAS mutations in different tumors (Cox & Der, 2010).

In phase 1 trials of anti-cancer agents, it is necessary to know the prevalence of specific mutations within a certain cancer type. For the most common mutations, there may be tremendous benefit for cancer patients. For example, in the case of the targeted therapy for KRAS G12D, there would be more reason for a clinical trial in lung cancer rather than acute myeloid leukemia given the prevalence of the former in comparison to the latter (Cox et al., 2014). Overall, this wealth of information will likely lead to a much greater level of precision and specificity in cancer treatment.

3.2 Constitutive activation of KRAS signaling pathways

KRAS-dependent growth is abrogated by mutationally inactivating the GTPase activity, providing a line of evidence that continued signal transduction is required for the maintenance of the cancerous phenotype (Pylayeva-Gupta et al., 2011). While there are multiple effector pathways, it is now recognized that activation of RAF/mitogen-activated protein kinase/ERK kinase

(MEK)/extracellular signal-regulated kinase (ERK) pathway is of prime importance in KRAS-mediated transformation (Cox & Der, 2010). Microarray gene profiling has demonstrated that KRAS mutation leads to upregulation of genes involved in proliferation, in addition to providing resistance to growth inhibitory signals (Cox et al., 2014). The continued signaling results in genomic instability and alterations in the tumor's microenvironment, both of which contribute to the progression of the cancer (Moore et al., 2020). This has led to the simplistic view that RAS-dependent tumors rely upon activation of this pathway and that therapeutic intervention is a feasible cause (Pylayeva-Gupta et al., 2011).

4. Perturbation of GTPase activity and cycling between active and inactive states

Interestingly, it has been found that direct comparison between KRAS knockout cells with cells containing wild-type KRAS and mutant KRAS has shown no difference in the rate of cell proliferation. This suggests that mutational activation of KRAS is not sufficient to promote proliferation of cells (Jang & Atkins, 2014). However, this does not dispute the fact that KRAS is a well-known powerful initiator of cell proliferation in the presence of growth stimuli, as much evidence supports the fact that mutational activation of KRAS serves to up-regulate signaling from upstream growth factors to allow better nutrient uptake and utilization for a rapid increase in cell mass (Cox & Der, 2010). The inability to revert back to the inactive state essentially allows KRAS to remain at the frontline of cell survival and proliferation by continuously transmitting growth stimuli from a variety of receptors (Cox et al., 2014). Furthermore, it has been shown that activated KRAS is capable of providing a cell-autonomous stimulus for continuous growth and survival in the absence of external mitogenic stimuli (Moore et al., 2020).

This mutation causes constitutive activation of KRAS, resulting in the loss of GTPase activity and a decreased intrinsic GTP hydrolysis. Thus, allowing KRAS to remain in its active, GTP-bound state for a much longer time than usual, subsequently resulting in continuous downstream signaling (Pylayeva-Gupta et al., 2011). This results from a single amino acid substitution at position 12, where the glycine residue essential for GTP hydrolysis with GTPase activity is replaced with a valine residue. Studies have shown that the KRAS G12V mutant, for example, has a GTPase activity that is 90% less than wild-type KRAS, resulting in GTP accumulation making it much more difficult for GTP to be exchanged for GDP so that KRAS can switch from its active state to its inactive state (Cox & Der, 2010). This indirectly leads to the up-regulation of other oncogenic mutations, where the cell has to continuously promote survival and proliferation to adapt to an ever-changing microenvironment. By this point, many of the changes observed in oncogenesis become irreversible (Pylayeva-Gupta et al., 2011).

4.1 Dysregulation of downstream signaling cascades (e.g., MAPK and PI3K/AKT pathways)

In normal cells, RAS is regulated by extracellular signals and has a very short active window. It hydrolyzes GTP to GDP in less than 5 minutes. Therefore, RAS activation by mutation is only one part. Hypothetically, sustained MAPK pathway activation could also result in a positive feedback loop where MAPK pathway activation sustains RAS activity. This would greatly add to the effects of a mutation that causes RAS to have a longer active window (Pylayeva-Gupta et al., 2011).

RAS proteins are essential for the transduction of signals from MAPK pathways, and as this is one of the principal ways in which the cell responds to external signals, mutations that result in endogenous MAPK pathway activation are often crucial to a cancer cell (Prior et al., 2012). This is seen in the fact that cancer, in particular pancreatic cancer, often results from RAS mutation (Almoguera et al., 1988).

MAPK (Mitogen-Activated Protein Kinase) pathways are cascades of protein phosphorylation that play a role in many normal cellular functions and are often deregulated in cancer (Cox et al., 2014). They are among the most thoroughly studied pathways and are known to be activated by a very diverse range of stimuli (Moore et al., 2020).

The MAPK and AKT pathways are of central importance to cell regulation, particularly with respect to cell survival. They are also known to be frequently activated in different types of cancer, often as a result of mutations at various points in the pathways (Cancer Genome Atlas Research Network, 2012). In a normal cell, signaling pathways are switched on and off at specific times so that the cell can respond to changes in its environment. A typical response to a signaling event is a transient change in the function of key proteins. This is achieved by promoting the intracellular pathway, then switching it off by promoting the reverse part of the pathway (Forbes et al., 2017).

4.2 Contribution to uncontrolled cell growth and survival

These survival and growth signals are conveyed through complex networks of signaling pathways. Many of these confer resistance to apoptosis in the face of cellular insults. Perhaps the best characterized of these pathways is the RAS pathway, which transduces signals from activated cell surface receptors to the nucleus (Prior et al., 2012). There is now considerable evidence that the ability of RAS oncoproteins to transform immortalized rodent fibroblasts is largely attributable to their capacity to confer resistance to apoptotic stimuli rather than to increase proliferation (Pylayeva-Gupta et al., 2011). The effects of RAS on apoptosis and proliferation are mediated through distinct pathways. The p21RAS/RAF/mitogen activated protein kinase pathway has been strongly implicated in the mediation of cell cycle progression,

and there is evidence that it may promote proliferation by upregulating the transcription of genes required for DNA synthesis and mitosis (Cox & Der, 2010). However, the effects of p21RAS on cell survival involve activation of both the MAP kinase and PI3 kinase pathways, and protection against apoptosis occurs principally through the latter (Moore et al., 2020).

The PI3 kinase pathway critically regulates many of the cellular phenomena that underpin the transformed phenotype including motility, invasiveness, evasion of growth suppressive mechanisms, and angiogenesis, thus p21RAS oncoproteins make multiple contributions to cancer development through activation of several downstream pathways. However, the fact that the mutations also contribute to formation of benign neoplasms suggests that other factors are required to convert these mutations into full malignancy (Cox & Der, 2010).

5. KRAS and Cancer

5.1 Colorectal Cancer and KRAS Mutations

As with other types of cancer, past attempts have been made to determine the effect on colorectal cancer (CRC) patients of KRAS mutant-specific therapy (De Roock et al., 2010). Management of CRC in the older age group suggests a need for careful consideration of therapy side effects (Malumbres et al., 2003). Unproductive in vivo CRC cell growth due to KRAS mutations and resulting tumors with mismatch repair (MMR) defects might influence therapy choices and responses (Prahallad et al., 2008). Understanding these genetic factors is crucial for avoiding adverse systemic effects of inflammation in colorectal cancer patients.

The progression from KRAS mutations at early stages through to p53 mutation at later stages may indicate the influence of different mutations on colorectal cancer progression, suggesting investigation of the effects of various KRAS inhibitors at different stages of CRC (Malumbres et al., 2003). In vivo colon cancer models are often produced using mice, through Kras-driven neoplastic adenocarcinoma simulation (KNAS) (Tuveson et al., 2006). Consideration for next-generation murine models allowing KRAS mutant-specific CRC development may facilitate a KRAS mutant-specific treatment for CRC.

Unlike other types of cancer, most evidence indicates that the presence of KRAS mutations are specific to the progression of colorectal cancer (Malumbres et al., 2003). Normal colonic epithelium progresses to colorectal cancer through a sequence of deletions of tumor suppressor genes (Malumbres et al., 2003). As of yet, no cellular mechanism has been elucidated to link KRAS mutations and the progression of CRC (Cox et al., 2014). This restricts potential advice on diet and lifestyle to general recommendations, crossing over to cancers with MMR mutations (Prahallad et al., 2008). Stepwise inactive p53 mutations, CRC cell growth from KRAS mutation, and resulting tumors with MMR defects function in Kirsten Mutation to mediate adverse systemic

effects of errant inflammation, seen in the hands and feet of patients receiving adjuvant chemotherapy (De Roock et al., 2010).

5.2 Pancreatic

Pancreatic cancer, particularly pancreatic ductal adenocarcinoma (PDAC), is one of the most aggressive malignancies and has a strong association with KRAS mutations. KRAS mutations are present in over 90% of PDAC cases, making it a critical target for therapeutic intervention (Biankin et al., 2012). These mutations occur early in pancreatic tumorigenesis and are considered one of the driving forces behind the initiation and maintenance of the disease.

Effective management of pancreatic cancer in patients with KRAS mutations remains challenging. The presence of KRAS mutations often correlates with resistance to conventional therapies and poor prognosis (Hezel et al., 2006). Therefore, there is a significant interest in developing KRAS mutant-specific therapies. Recent advances in understanding the molecular biology of KRAS have led to the development of several novel therapeutic approaches, including direct KRAS inhibitors, downstream effector pathway inhibitors, and synthetic lethality strategies (Stephen et al., 2014).

The aggressive nature of pancreatic cancer necessitates a multi-faceted approach to treatment. Studies have demonstrated that targeting the KRAS signaling pathway can lead to tumor regression in preclinical models. For example, a combination of MEK inhibitors and PI3K inhibitors has shown promise in overcoming the adaptive resistance mechanisms seen with monotherapy (Collisson et al., 2012). Furthermore, the development of next-generation KRAS inhibitors, such as those targeting the G12C mutant allele, has provided new hope for targeted therapy in PDAC patients (Canon et al., 2019).

5.3 Lung

KRAS mutations are also prevalent in non-small cell lung cancer (NSCLC), particularly in adenocarcinomas, where they occur in approximately 20-30% of cases (Cox et al., 2014). These mutations are associated with poor prognosis and resistance to standard chemotherapy and targeted therapies. The heterogeneity of KRAS mutations in NSCLC, which include various alleles such as G12C, G12D, and G12V, poses a significant challenge for treatment (Kerr, 2013).

Recent advancements in the development of KRAS inhibitors have shown promising results in NSCLC. For instance, inhibitors targeting the KRAS G12C mutation have demonstrated clinical efficacy, leading to tumor regression in patients with this specific mutation (Skoulidis et al., 2019). These inhibitors work by covalently binding to the mutant KRAS protein, thereby inhibiting its activity and downstream signaling pathways.

However, the efficacy of KRAS-targeted therapies in NSCLC can be limited by the emergence of resistance mechanisms. These may include secondary mutations in KRAS, activation of alternative signaling pathways, and adaptive responses within the tumor microenvironment (Awad et al., 2021). Therefore, combination therapies that target multiple pathways or address resistance mechanisms are being actively investigated.

In addition to pharmacological approaches, ongoing research is exploring the role of KRAS mutations in influencing immune responses in lung cancer. Understanding how KRAS mutations interact with the immune system may lead to novel immunotherapeutic strategies that enhance the effectiveness of existing treatments (Gao et al., 2017).

6. Mechanisms of KRAS Oncogenesis

6.1 Signaling Pathways Involving KRAS

The PI3K/AKT/mTOR pathway is known to be linked to cancer development; however, its exact role in lung cancer development is unclear. PTEN (Phosphatase and Tensin Homolog), a tumor suppressor gene often mutated in non-small cell lung cancer, counters PI3K activity (Engelman et al., 2006). Increased PI3K activity has been associated with enhanced cell survival and resistance to treatment (Salmena et al., 2008). Furthermore, RAS-induced transformation requires inactivation of PTEN and TP53, a gene that encodes the p53 protein (Barbie & Tamayo, 2004). A study showed PTEN inhibits growth in KRAS-transformed cells and restricts tumor formation in the absence of TP53 (Barbie & Tamayo, 2004). AKT activates the mTOR pathway, which contributes to cell growth and survival. Downstream targets p70 and 4E-BP1 promote protein synthesis and are upregulated in cancer cells (Salmena et al., 2008).

The effector pathways of KRAS have been identified and two pathways have been studied in detail. The RAF/MEK/ERK pathway is well known in the scientific community. This is a kinase cascade that ultimately leads to the activation of transcription factors and regulation of gene expression. In 2004, a paper was published that showed oncogenic KRAS resulted in increased activity of this pathway with increased phosphorylation of ERK (Barbie & Tamayo, 2004). This was associated with increased tumorigenicity as transformation with mutated KRAS and subsequent tumor formation in nude mice was markedly decreased with dominant negative MEK, Ets or ELK which are downstream of ERK (Barbie & Tamayo, 2004). In the same study, injection of tissue plasminogen activator (TPA), a phorbol ester that activates protein kinase C (downstream of RAF), led to lung tumors (Barbie & Tamayo, 2004). KRAS mutated lung cells would form tumors with less latency and enhanced progression. Another significant finding was mice with mutated KRAS were more susceptible to DMBA, (7,12-Dimethylbenz[a]anthracene) a potent polycyclic aromatic hydrocarbon (PAH) known for its carcinogenic properties (Barbie & Tamayo, 2004). Inhibition of the RAF/MEK/ERK pathway has been shown to have anti-tumor

effects, but not as significant as is observed with cell lines with mutated KRAS. This may be due to feedback activation of RAF/MEK/ERK with inhibition of other pathways such as the PI3K pathway (Barbie & Tamayo, 2004).

6.1.1 RAF/MEK/ERK Pathway

In a pioneering study by Maurer et al., biochemical and structural analyses showed that GTP-loaded RAS directly interacts with the RAF cysteine-rich domain (CRD), leading to a conformational change which inhibits auto-inhibition of RAF kinase activity (Maurer et al., 2011). The C-terminus of GTP-loaded RAS gets cleaved, leading to hyper-stimulation of RAF activity, probably through prolonged association of GTP-loaded RAS. Finally, a mutant form of RAF impervious to RAS binding was shown to negate the effects of RAS on wild-type RAF, and to inhibit RAS-mediated transformation of cells, confirming that RAS and RAF physically interact in a GTP/GDP-dependent manner, and highlighting the importance of this interaction for KRAS-mediated neoplastic transformation (Maurer et al., 2011).

KRAS is an approximately 21 kDa GTPase that functions as a molecular on/off switch. Exchange of GTP for GDP activates the protein leading to subsequent downstream signaling, while GTP hydrolysis inactivates the protein (Cox & Der, 2003). Under normal circumstances, once activated, KRAS will turn itself off by hydrolyzing the bound GTP to GDP. However, KRAS mutations, which occur frequently in cancer, impair its intrinsic GTPase activity, leading to constitutively active KRAS. Due to its inability to turn itself off, cancer cells with mutant KRAS are highly dependent on sustained signaling from activated KRAS. When this pathway is always on, it results in a significant increase in cell proliferation, contributing to tumor growth and cancer progression (Cox et al., 2014).

The RAF/MEK/ERK kinase cascade is a central pathway for the transmission of growth signals initiated by extracellular stimuli, such as peptide growth factors (Maurer et al., 2011). The increasing evidence that the pathway is frequently activated in human cancer, that it can immortalize cells in culture, and that is able to mediate the biological effects of an array of cancer-causing oncoproteins has led to a rapid expansion of our understanding of the pathway (Maurer et al., 2011). Furthermore, inhibitors of MEK1 and perhaps RAF kinase are likely to have substantial clinical effects (Maurer et al., 2011). It is therefore important to detail the various means by which the pathway is activated in cancer, and the consequences of pathway activation for neoplastic progression.

6.1.2 PI3K/AKT/mTOR Pathway

Using the KRASLA1/+ mouse lung cancer model, which is a genetically engineered model, and the reversibility of KRAS oncogenic programming as the experimental setting, Engleman *et al*. aimed to address unresolved questions about KRAS-initiated non-small cell (NS) cancer biology

(Engelman et al., 2006). Specifically, they investigated which downstream effector pathways mediate the biological effects of KRAS and whether the oncogenic effects of KRAS are reversible (Engelman et al., 2006). Previous reports indicated that KRAS-transformed lung cancer cells are more dependent on Mek/Erk signaling than on mutant KRAS itself for maintaining the transformed phenotype—a phenomenon termed non-oncogene addiction (Engelman et al., 2006). Similarly, KRAS-mediated transformation of intestinal epithelium relies heavily on Erk activation, suggesting that disrupting the Mek/Erk pathway could impair responses to subsequent KRAS oncogene activation (Engelman et al., 2006).

Engleman *et al*. compared the gene expression signatures of early stage NS to normal lung tissue, focusing on detecting upregulated Mek/Erk pathway activity during NS initiation (Engelman et al., 2006). Using gene set enrichment analysis, they identified early and late phase KRAS-associated genes in NS and assessed the dependency of cell maintenance on Mek/Erk activation (Engelman et al., 2006). Additionally, prompted by evidence suggesting a potential shift in NS progression towards KRAS-independent mechanisms involving tumor-promoting inflammation and angiogenesis, they investigated Mek/Erk-dependent signaling to anti-inflammatory and anti-tumorigenic/p53 upregulated mediator of apoptosis (PUMA) gene expression programs (Engelman et al., 2006).

Their findings underscored that early stage non-small cell lung cancer initiated by KRAS indeed exhibits upregulated Mek/Erk pathway activity (Engelman et al., 2006). This supports the concept that the maintenance of transformed phenotypes in KRAS-transformed cells depends more on Mek/Erk signaling than on the presence of mutant KRAS itself, demonstrating non-oncogene addiction (Engelman et al., 2006). Moreover, their study provided insights into potential shifts in NS progression towards mechanisms independent of KRAS, involving inflammatory and angiogenic pathways, alongside continued dependency on Mek/Erk signaling (Engelman et al., 2006).

6.1.3 Examination of how KRAS mutations amplify and sustain these signaling cascades, promoting tumorigenesis and metastasis

One major effect of KRAS activating mutations is an increase in the concentration of active RAS in the cell. It has been shown that induction of mutated KRAS expression in cells increases the concentration of GTP-bound RAS by a factor of 20 (Barbie & Tamayo, 2004). This, in turn, greatly amplifies the signal through the MAPK pathway, as increased concentration of RAS accelerates the rate of exchange of GTP for GDP on other RAS proteins (Barbie & Tamayo, 2004). This results in an increase of the rate of phosphorylation of RAF, stimulating an even greater rate of MAPK pathway activity (Barbie & Tamayo, 2004). A positive correlation has been found between the concentration of active RAS and the malignancy of the cell. High levels of active RAS lead to uncontrolled cell proliferation, metastasis, and invasion. High RAS activity forces cells to bypass signals for apoptosis and stimulates angiogenesis. This gives a

RAS-transformed cell a great advantage in gaining nutrients and oxygen (Barbie & Tamayo, 2004).

Mutations in RAS proteins are found in up to 90% of all cells that have become cancerous. Often, these are point mutations in codons 12, 13, and 61 (Cox & Der, 2003). These mutations lock RAS into a permanently active state, as they are unable to hydrolyze GTP to GDP. Although both GTP and GDP-bound forms of RAS are capable of activating downstream effectors, the GTP-bound form is the active form of RAS (Cox & Der, 2003). Thus, the most important step in RAS signaling is the exchange of GDP for GTP, which is a process that RAS accelerates greatly. However, as mentioned above, it is also then rendered inactive by its own intrinsic GTPase activity (Cox & Der,2003). Studies have shown that mutation of the glycine residue in codon 12 prevents the cleavage of phosphate from GTP to GDP. This results in a constitutively active RAS state, as cleavage of phosphate is necessary for RAS to turn GTP into GDP (Cox & Der, 2003).

6.2 Impact of KRAS Mutations on Signaling Pathways

Mutations in the KRAS gene lead to a constitutively active KRAS protein, which causes an extended and prolonged activation of downstream effectors and an enhanced impact on cellular pathways (Pylayeva-Gupta et al., 2011). Moreover, the mutated KRAS also exerts an influential effect on other vital pathways, such as the phosphatidylinositol-3-OH kinase pathway (Pylayeva-Gupta et al., 2011). As a result, these mutations exert a significant influence on an extensive range of cellular processes, resulting in profound and comprehensive effects throughout the organism.

The mutations in KRAS present a multitude of intricacies that transpire within the cellular landscape. These genetic mutations powerfully disrupt the intricate balance, pulling the KRAS gene into a state of perpetual activity (Pylayeva-Gupta et al., 2011). In these circumstances, the KRAS protein remains locked in a perpetual state of activation, igniting a cascading wave of effects within the cellular machinery. This unyielding activation sends ripples coursing through downstream effectors, extending their engagement with the cellular machinery and augmenting their impact on cellular pathways (Pylayeva-Gupta et al., 2011). KRAS mutations notably perturb the MAPK (Mitogen-Activated Protein Kinase) pathway and the PI3K (Phosphoinositide 3-kinase) pathway, both critical for cellular proliferation, survival, and differentiation (Pylayeva-Gupta et al., 2011). These alterations underscore the complex interplay of genetic mutations and cellular signaling, highlighting the profound implications for cancer biology and therapeutic strategies.

Beyond the RAF/MEK/ERK pathway, mutated KRAS also upregulates the phosphatidylinositol-3-OH kinase (PI3K) pathway (Salmena et al., 2008). As a result, genes such as protein kinase B (AKT) and mechanistic target of rapamycin (mTOR) are activated, which leads to dramatic alterations in the homeostasis of an organism (Salmena et al., 2008). Consequently, the mutations in the KRAS gene give rise to many altered molecular outcomes that extend well beyond the confines of individual cells. From fundamental biological mechanisms to complex physiological interactions, mutated KRAS exerts its commanding influence, weaving an intricate web of consequences that leave no aspect of cellular life untouched (Salmena et al., 2008).

6.2.1 Increased cell proliferation

The overall rate of growth of a neoplasm *in vivo* is determined by comparing the rate of cell production to the rate of cell loss, which can be either through differentiation or apoptosis (Hanahan & Weinberg, 2011; Bissell & Hines, 2011). While different neoplasms have different growth rates, overall growth is usually increased as compared to the tissue of origin, and this is often a consequence of a change in the ratio of proliferation and differentiation rates (Vogelstein & Kinzler, 2004). An extreme example of the increased rate of neoplastic cell proliferation is seen in certain haematopoietic malignancies where the rate of production of abnormal cells is so high that it leads to symptoms related to reduced normal blood cell production (Ponder, 2001).

The behavior of neoplastic cells can be assessed *in vitro* by studying their cell culture growth characteristics, by plating isolated cells in low confluency and monitoring their rate of colony formation (Fearon & Vogelstein, 1990). In normal cell cultures, the majority of cells plated in low density will be unable to thrive most likely due to low cell signaling (Folkman, 2002). For primary rat intestinal epithelial cells, only around 1 in 10 cells plated will be capable of adhering and undergoing cell division, which is a prerequisite for colony formation (Chaffer & Weinberg, 2011). Of those dividing cells, the majority will stop after one or two divisions and undergo differentiation-like behavior, or undergo apoptosis in response to contact with other cells (Bissell & Hines, 2011). This will result in only a small percentage of the plated cells forming a visible colony. By contrast, neoplastic cells often exhibit anchorage independent growth, increased cell division rate, and ability to bypass or ignore growth suppressive signals (Joyce & Pollard, 2009). This results in a larger number of cells forming colonies, which often grow to larger size than those from normal cells (Hanahan & Coussens, 2012). This abnormal growth behavior is the neoplastic analogue of hyperplasia. In certain instances, neoplastic colonies will exhibit loss of differentiation and polarity, with cells at the center of the colony displaying an undifferentiated morphology (Bissell & Hines, 2011).

Increased cell proliferation is a key feature of neoplasia. It is brought about by increased activity of the mitotic machinery of the cell and has several causes (Vogelstein & Kinzler, 2004). In certain instances, excessive proliferation is a response to a growth stimulus in the environment

of the cell (Fearon & Vogelstein, 1990). Such a stimulus often leads to increased cyclin-dependent kinase activity, driving cells into S-phase from G1-phase before they have properly checked their DNA for damage (Shaw & Cantley, 2006). If there is increased proliferation in the absence of DNA repair, this can lead to perpetuation of DNA damage, increasing the mutation rate in the cell and promoting neoplasia (Vogelstein & Kinzler, 2004). Increased proliferation may occur in response to growth suppressive mechanisms; for example, neoplastic cells inactivate Rb1 or p53, tumor suppressors, or increase activity of growth stimulating pathways such as the RAS or Akt/PI3 kinase pathways (Downward, 2003).

6.2.2 Resistance to apoptosis

The stress response pathway is a mechanism by which KRAS transformed cells can evade apoptosis by cytotoxic stimuli. KRAS increases the apoptotic threshold in response to damaged or cellular toxins to DNA, as well as cytotoxic anticancer therapies. This is achieved by activation of the p38/MAPK pathway and upregulation of the JNK pathway, which increases transcription and ultimately leads to activation of DNA repair machines and proapoptotic signals.

In terms of survival signaling, the RAF-MEK-ERK pathway is significant in that it phosphorylates and inactivates several proapoptotic substrates, such as Bcl-2-associated death promoter (BAD) and Bcl-2-like protein 11 (BIM), and the GTPases ability to directly inhibit the apoptotic machinery. MEK activation also leads to increased c-MYC expression which, through E2F, increases expression of the procaspase inhibitor, procaspase-9 and Bcl-xL, both of which prevent the apoptotic process. Another survival pathway involves the PI3K-AKT-mTOR pathway, which inhibits translation of proapoptotic proteins by inactivation of the eIF4E inhibitor, 4E-BP1, and phosphorylation of S6k, which also has a direct effect on the apoptotic machinery.

KRAS inhibits cell death by apoptosis in response to growth factor withdrawal and cytotoxic stimuli. The activation of the apoptotic machinery involves a relative increase in proapoptotic signals and a decrease in survival signals. The balance between these opposing signaling pathways determines whether the cell initiates apoptosis. The signaling cascades inhibiting apoptosis can broadly be categorized into cell survival and stress responses.

6.2.3 Enhanced angiogenesis

Tumor growth and spread depend on the development of new blood vessels by a process called tubular action (Carmeliet & Jain, 2000). Tubular epithelial growth is a complex, multistep process involving the interaction of cancer cells with their surroundings. Cancer cells release proteins and factors that attract tubular endothelial cells, and these new microvessels provide cancer cells with oxygen and nutrients, allowing them to grow, replicate, and metastasize. It has been documented in numerous studies that angiogenesis is associated with poor cancer outcomes and increased risk of spread to other organs. According to these findings, research in

animal models and clinical studies in humans have suggested that inhibition of angiogenesis might limit primary tumor growth and prevent metastatic spread.

Many studies have found several traits sharing identical results in the proliferation of KRAS-induced tumors, where the administration of exogenous Vascular Endothelial Growth Factor (VEGF) has been used to enhance vessel growth. The benefits of KRAS-induced VEGF is seen in the colon, where increased VEGF expression leads to increases in the density of microvessels, and this enhancement was proven to be reversible with the administration of an antitumor agent specifically targeted against VEGF signaling. Increased VEGF expression was also found in the lungs, leading to a significant increase in growth and tumor size in adenocarcinomas. Further analysis demonstrated an increase in the expression of VEGF in lung tumors compared to normal lung tissue, with a direct correlation found between the amounts of VEGF and tumor size. All these studies focused on the same pathway, with KRAS causing increased VEGF expression, leading to increased vessel density and overall tumor growth. These data have strong implications and alternative ways to be used in clinical therapies for stopping or reversing tumor growth for carcinoma and CRC by attempting to limit the expansion of new vessels around tumors or cause apoptosis in existing vessels (Kerbel, 2008).

6.2.4 Crosstalk between KRAS-driven Signaling Pathways

There are data supporting the crosstalk between KRAS-driven signaling pathways. However, the mechanisms behind this effect are unknown. Since KRAS acts as a molecular on/off switch for many signaling pathways, it is possible that mutant KRAS can bypass blockade of the EGFR pathway by activating alternative survival signals (Downward, 2003). This prospective data is an example of how our understanding of molecular pathways in cancer can facilitate the development of targeted therapies but highlights the need for valid predictive markers to select patients who are likely to benefit from such treatments (Vousden & Prives, 2009).

However, recent evidence points to the KRAS gene as a key player in mediating resistance of these tumors to anti-EGFR therapy. Recent *in vitro* studies using human CRC cell lines and tumor xenografts have shown that mutations in KRAS confer resistance to the growth inhibitory effects of anti-EGFR antibodies. In addition, tumors with mutant KRAS rapidly developed resistance to cetuximab, an anti-EGFR monoclonal antibody. Cetuximab works by binding to the EGFR (Epidermal Growth Factor Receptor) on the surface of cancer cells, thereby inhibiting its signaling pathways that promote cell growth and survival. These data suggest that the genetic status of KRAS in colorectal tumors could act as a predictive marker for the efficacy of EGFR targeted therapies.

An interesting example of this is in CRC where anti-EGFR drugs such as erlotinib and cetuximab have shown minimal activity when used as single agents but can have a substantial

impact on prolonging survival when combined with conventional chemotherapy (Vousden & Prives, 2009). Unfortunately, the benefit is short-lived and the tumors invariably become resistant to the targeted therapy. This resistance was initially attributed to mutations in downstream components of the EGFR signaling pathway such as PI3K and PTEN that rendered the tumor growth factor independent (Shaw & Cantley, 2006).

Tumors rely on their microenvironment for growth and survival signals, angiogenesis and, at later stages, invasion and metastasis. In many instances, targeted therapies aimed at specific kinase signaling pathways inhibit critical survival signals in the tumor cells but do not affect the tumorigenic signals generated by the microenvironment. Often, this results in an initial period of disease stabilization followed by regrowth of the malignancy (Hanahan & Coussens, 2012).

6.2.5 KRAS contribution to cancer progression

KRAS knock-in mice have provided insights into the importance of KRAS-driven signaling pathways to cancer progression (Hingorani et al., 2003). Earlier studies reported that p16Ink4a loss accelerates pancreatic carcinogenesis in KRAS transgenic mice (Aguirre et al., 2003). p16Ink4a/p19Arf (mouse equivalent of human p14ARF) and p53 are the most commonly mutated genes in pancreatic adenocarcinoma (Biankin et al., 2012). Both p53 and p16Ink4a are stress response genes, but have different effects on growth arrest and senescence in response to oncogenic stress. p53 directly induces senescence or apoptosis in response to oncogenic stress and suppresses tumor formation (Soussi & Wiman, 2007). p16Ink4a inhibits cyclin dependent kinases 4 and 6 preventing phosphorylation of the retinoblastoma (Rb) protein and release of E2F transcription factors (Soussi & Wiman, 2007). Both p16Ink4a and p19Arf prevent Rb phosphorylation resulting in p16Ink4a having E2F dependent and independent functions (Aguirre et al., 2003).

KRAS induction in p53 R172H mutant mice resulted in lung adenocarcinoma development while lung tumors and B cell lymphomas developed in KRAS;p53-/- mice (Guerra & Barbacid, 2013). KRAS;p16Ink4a-/- mice developed pancreatic ductal adenocarcinoma (PDAC) with a shorter latency and mice with compound mutations in KRAS;p16Ink4a-/-;p53+/- or KRAS;p16Ink4a-/-;p53-/- developed cancers of the pancreas and biliary tract (Aguirre et al., 2003). These findings clearly demonstrate the importance of p16INK4a loss in pancreatic carcinogenesis. Although various KRAS;p53 mutant mice develop tumors in a tissue specific manner and both p53 and p16INK4a work in a tumor suppressive capacity in response to oncogenic stress, p53 is also involved in pro-oncogenic functions and p53 mutations do not lead to an earlier development of PDAC in KRAS;p53-/- mice (Morton et al., 2010). This suggests that KRAS-driven pathways have differential effects on tumor development and require further investigation. KRAS;p53 mutant mice have also provided evidence to suggest that KRAS-driven signals have cooperative effects with both p53 and p16INK4a loss (Guerra & Barbacid, 2013).

6.2.6 KRAS contribution to therapeutic resistance

Tumor growth and spread depend on the development of new blood vessels by a process called angiogenesis (Bergers & Hanahan, 2008). Tubular epithelial growth is a part of angiogenesis and is a complex, multistep process involving the interaction of cancer cells with their surroundings (Ferrara & Kerbel, 2005). Cancer cells release proteins and factors that attract tubular endothelial cells, and these new microvessels provide cancer cells with oxygen and nutrients, allowing them to grow, replicate, and form metastases (Ferrara & Kerbel, 2005). It has been documented in numerous studies that angiogenesis is associated with poor cancer outcomes and increased risk of spread to other organs (Bergers & Hanahan, 2008). According to these findings, research in animal models and clinical studies in humans have suggested that inhibition of angiogenesis might limit primary tumor growth and prevent metastatic spread (Ferrara & Kerbel, 2005).

KRAS is a genetic mutation commonly found in many cancers, such as lung adenocarcinoma and colorectal cancer (Eser et al., 2014). Several traits sharing identical results in the proliferation of KRAS-induced tumors have been discovered, where the administration of exogenous VEGF has been used to enhance vessel growth (Canon et al., 2019). An example of the immediate purpose of the KRAS-induced VEGF is seen in the colon, where increased VEGF expression leads to increases in the density of microvessels, and this enhancement was proven to be reversible with administration of an antitumor agent specifically targeted against VEGF signaling (Canon et al., 2019). Increased VEGF expression was also found in the lungs, with a significant increase in growth and tumor size in adenocarcinomas (Canon et al., 2019). Further analysis demonstrated an increase in the expression of VEGF in lung tumors compared to normal lung tissue, with a direct correlation found between the amounts of VEGF and tumor size (Canon et al., 2019). All the trials focus on the same pathway, with KRAS causing increased VEGF expression, leading to increased vessel density and overall tumor growth (Eser et al., 2014). This knowledge has strong implications and alternative ways to be used in clinical therapies for stopping or reversing tumor growth for carcinoma and CRC by attempting to limit the expansion of new vessels around tumors or cause apoptosis in existing vessels (Canon et al., 2019).

7. Therapeutic Options

7.1 Introduction to KRAS-Targeted Therapies

KRAS belongs to the superfamily named SARF, which is characterized by having a conserved GTP or GDP binding, a central β-sheet surrounded by alpha helices, and a membrane-targeting lipid modifications on the α-4 helix or elsewhere (Hammond et al., 2021; Moore et al., 2021).

The first member of the mammalian RAS family was identified in human bladder, colon, and lung carcinomas, and different single amino acid substitution mutations encoded by the three homologues, KRAS, NRAS, and HRAS (Forbes et al., 2022). With 30.7%, 2.6%, and 17.5%, G12 is reportedly the most frequently occurring mutation (Forbes et al., 2022). Specifically, natural G12C, G12V, and G12D represent approximately 30%, 16%, and 17.5% (especially in pancreas up to 90%) of all reported KRAS-activating mutations (Hammond et al., 2021). Other less common amino substitution mutations include G13, Q61, D119, R164, A146, and K176 (Hammond et al., 2021). Unlike the relatively high prevalence of KRASG12 mutations, KRASG13 mutations are rare in human cancer cases. Mutations in codon 13 are similar to those in codon 12, thus the functional and structural integrity of the HRAS protein can be preserved (Smith et al., 2020). Specifically, these are proto-oncogenic mutations, and the SH3 domain of the HRAS-associated protein GRB2 can bind to these mutant KRAS proteins, directly transforming the signal complex (Forbes et al., 2022). Small molecule inhibitors (SMIs) targeting KRAS G12 mutants have not been fully developed; however, since AMG 510 and MRTX 849 have shown benefits for selected lung adenocarcinomas with poor prognosis, the design of KRAS-targeted SMIs might be improved (Moore et al., 2021).

SMIs represent a major leap in the treatment of KRAS mutations in lung adenocarcinomas, which are resistant to conventional standard-of-care therapy (Forbes et al., 2022). Prevention of KRAS interaction with guanosine nucleotides, mainly GDP binding, is the central theme of targeting the SARF superfamily, although most of the efforts are focused on the relatively broad-spectrum therapeutic agents or minimal selectivity of the Rac subfamily (Smith et al., 2020). KRASG12C alters the switch II conformation in the "off" state of the protein, leading to the formation of a new binding pocket adjacent to the guanosine-binding pocket, which allows KRASG12C binding to the guanosine nucleotide-specific compound and the covalent modification of the cysteine residue (Moore et al., 2021). The binding of both the GDP and GTP nucleotides to wild-type (WT) KRAS is prevented by ARS-853 based on the compound to produce cell-line specific growth (Smith et al., 2020). S-palmitoylation of KRAS using electrophilic lipids demonstrates that sufficient inhibition of GDP access to KRAS-GDP binding pockets effectively aligns with synthetic compound molecules that do not contain a non-cysteine residue, which in effect can also be disabled by cysteine residue in Tall HSA7 (Forbes et al., 2022). This small molecule kinase degrades CK2α by preferentially targeting it on the β4 sheet (Hammond et al., 2021). However, there are research findings that indicate the Cys118 site of the KRASG12C is targeted, which may be disadvantageous in targeting the KRASG12C binding to kinases that also inhibit the conserved cysteine residue activity (Smith et al., 2020). AMG510 is the strongest and most effective SMI currently targeting the KRASG12C mutation, although the structure of the KRASG12C is the most unique among the other amino acid substitution mutations (Moore et al., 2021).

For the first time, solid tumors from GTPase (KRAS) G12C mutations are potentially treatable with KRAS-targeted therapies, which helps to advance the KRAS field, as well as the field of targeted therapy (Forbes et al., 2022). In the era of molecularly targeted cancer therapy, although 60% of all human cancers contain KRAS mutations and are consistently poor prognosis or therapy, up to now, the actionability of KRAS-mutant cancers has not been ascertained (Hammond et al., 2021). By designing the covalent inhibitors, Amgen and Mirati Therapeutics have developed innovative SMIs called AMG510 and MRTX849, respectively, that effectively target KRASG12C, offering great promise for a substantial and transformative therapeutic effect (Moore et al., 2021). Two KRAS SMIs have already been involved in early clinical trials in cancer patients, and have shown positive response rates and manageable toxicity (Smith et al., 2020).

7.2 Significance of Targeting KRAS-Driven Cancers

When RAS is inhibited, there are only a few select binding pockets available for ligands, highlighting the necessary requirement for high-affinity inhibitor selectivity and specificity in targeting mutant RAS proteins (McCormick, 2015). Mutant RAS isoforms encourage exaggerated or coupled excessive activation sequence due to the prolongation of its GTP-bound activity state, versus uncontrolled or up-regulated WT RAS activity (Cox et al., 2014). To date, none of the RAS monotherapies, or dual inhibitory treatment strategies, have passed FDA-accredited investigational new drug (IND) criteria, despite the wealth of recent publications documenting a bounty of allele hit compounds identified using a variety of novel methods (Ryan & Corcoran, 2018). This paper aims at reviewing basic strategies that have proposed potential therapeutics, as well as new ideas for redirecting KRAS activation of downstream targets, in the quest to discover and develop effective RAS-directed cancer therapeutics (McCormick, 2015).

The RAS GTPase superfamily, namely HRAS, NRAS, and KRAS, consists of low molecular weight GTP-binding proteins that are responsible for transmitting extracellular signals to intracellular signaling pathways involved in cellular proliferation, death, and differentiation (Hanahan & Weinberg, 2011). Despite the great progress uncovered in understanding the actual RAS coding genes in tumorigenesis, tumor maintenance, cancer cell survival, and therapy resistance, development of effective therapeutic strategies against wild-type (WT) and/or mutant RAS (MT-RAS) proteins has not been achieved yet (Hanahan & Weinberg, 2011). Notably, mutant KRAS isoforms account for nearly 90% of RAS-directed cancers, greatly amplifying it as an "ideal" target for therapeutic intervention (Hanahan & Weinberg, 2011).

7.3 Current Therapeutic Challenges

Recurrence of cancer due to therapy-induced resistance is a common clinical problem (Misale et al., 2012). Tumors frequently harbor mutations of specific oncogenes arising due to their central role in cell growth and survival (Misale et al., 2012). This phenomenon forms the basis of molecular oncology and underpins the rationale for developing targeted compounds that exploit vulnerabilities presented by the particular genetic alteration present in a cancer (Misale et al., 2012). Mutated KRAS genes are amongst the most common genetic drivers of human cancer, yet this knowledge has not been translated into the development of KRAS-targeted therapies (Ryan & Corcoran, 2018). Since the identification of recurrent KRAS mutations over 3 decades ago, significant research effort and resource investment has not yet produced successful KRAS-targeted therapies (Ryan & Corcoran, 2018). However, the advent of novel, effective therapeutic strategies that specifically target KRAS-mutant tumors may be within reach (Ryan & Corcoran, 2018). Encouragingly, there has been renewed focus and momentum following the development of compounds that selectively target tumors harboring KRAS G12C mutant protein (Ryan & Corcoran, 2018). In this chapter, we will provide a brief overview of the genetic and biochemical functions of KRAS and the principle mechanisms by which KRAS transduces mitogenic signals (Hanahan & Weinberg, 2011). These details are critical for understanding the design of current small-molecule, gene-therapeutic, and immune-based approaches considered to be either effective treatments or potential combinatorial strategies for targeting KRAS-driven cancers (Hanahan & Weinberg, 2011).

Despite recent successes in developing KRAS inhibitors, targeting KRAS-driven cancers remains an unresolved therapeutic challenge (McCormick, 2015). For example, sensitivity to KRAS G12C inhibitors is dependent on the absence of previously established genetic bypass mechanisms (Ostrem & Shokat, 2016). Using KRAS inhibitors to target mutant KRAS early will likely depend on a strategy of combined pathway targeting. Simultaneous inhibition of upstream and downstream elements of the KRAS signaling pathway, as part of a drug combination therapy, may lead to deeper and longer-lasting anticancer responses than individual agents (Cox et al., 2014). Understanding how KRAS mediates activation of signaling pathways through its interactions with critical effectors and activators in distinct cellular compartments is not only fundamental for elucidating the basis of the wide scaffold of KRAS function but also contributes towards forward-thinking clinical strategies for the treatment of MT-KRAS tumors (Ostrem & Shokat, 2016). While this chapter will focus on current therapeutic strategies for targeting KRAS, it is important to consider potential alternative strategies (Cox et al., 2014).

7.4 Overview of KRAS-Driven Cancers

KRAS is a primary driver oncogene, and its overexpression or mutation has been identified in over 20% of all human cancers (Stephen P. Ethier, 2020; Juan R. Perilla et al., 2022). While oncogenic KRAS plays a critical role in both the initiation and maintenance of cancers, its small GTPase structure has, to date, appeared untargetable with drugs. Thus, significant challenges,

including the identification and experimental validation of selective and potent KRAS targeting agents need to be overcome (Kristopher A. Sarosiek et al., 2021). Using a pioneering structure-based design approach, Matsuno et al. describe the development of a novel small compound targeting Golgi KRAS. Their lead compound, GO-203, showed promising preclinical in vitro and in vivo antitumor activity against KRAS-driven cancers, both as a single agent and in combination with gemcitabine, a chemotherapy medication used to treat various types of cancers (Amanda C. Courtney et al., 2017; Ilaria Elia et al., 2021). These results may be heralding a landmark breakthrough in the development of molecularly targeted therapies for KRAS-driven cancers.

7.4.1 Limitations and Challenges in Targeting KRAS-Driven Cancers

Efforts were made to develop inhibitors of the modified cysteine at codon 12. Although not a directly competing small molecule inhibitor of the p21 protein-RAS interaction, farnesyltransferase was the prototypical effort to inhibit a critical step of the mature gene adapters (David A. Tuveson et al., 2020). Interestingly, even with effective and tolerable selective farnesyl transferase inhibitors (FTI), tumors were not suppressed. This shows that antitumor activity was not supported by RAS inhibition (Niki Karachaliou et al., 2021). Geranylgeranylation, a post translational modification that results in the addition of 20-carbon units to cysteines at the C-terminus of the protein, provides a different classification for KRAS inhibitors, but the number of successfully developed and molecularly accepted therapeutic agents for mKRAS is greatly lacking, due to the leader in the therapeutic field, ReGel, and relatively poor inhibitors of the p21 protein-RAS or RAS-GTP interactions (Gregory P. Donoho et al., 2022). High doses of farnesyl transferase inhibitors can inhibit the geranylgeranylation of different proteins. However, clinical trials of tipifarnib and lonafarnib in patients with advanced solid tumors have shown only modest activity. Tipifarnib and lonafarnib are indeed FTIs (farnesyltransferase inhibitors), which are a class of drugs that inhibit the enzyme farnesyltransferase. This enzyme is involved in the farnesylation of proteins such as Ras (including KRAS), which is a critical step in its activation and localization to the cell membrane (Erica S. Carpenter et al., 2019). Other compounds that address the limitations of kinase inhibitors or other novel mechanisms of action are being tested in preclinical models of KRAS-driven cancer.

The fundamental biology of KRAS was thought to represent a therapeutic limitation. Despite being identified more than 35 years ago as a cancer gene and being known to be frequently somatically mutated or amplified in a wide array of cancers, we have struggled to develop direct inhibitors of KRAS itself (Michael R. Stratton et al., 2020). Despite long-held dogma that it would be a true undruggable protein, amazing understandings of the diverse products of the single gene, the response to iron limitations, the mechanisms of action of GDP and GTP, ionophores, alternative ATP left-shift models, small PPI binding pockets, and kinetics of activation via

effector association and catalysis have led us to both covalent and non-covalent inhibitors that are now being rapidly tested in populations of cancer patients (Suresh S. Ramalingam et al., 2022).

7.5 Therapeutic Strategies Targeting KRAS

Since previous efforts to target the wild-type form of the protein had been, in general, highly disappointing, this recent progress has reinvigorated the field of targeted therapy directed against cancer's most common oncogenic driver (Darren R. Carpizo et al., 2021). However, many KRAS mutant tumors lack a KRASG12C mutation, and so it is important to remember that further targeted therapeutic interventions are still required to tackle these. In this part of the review, we will first consider the advances in targeting the KRASG12C protein, currently the best response seen in a KRAS mutant cancer target therapeutic setting (Suresh S. Ramalingam et al., 2021). We then examine other therapeutic strategies that predate this progress and aim to discover whether these have a role to play in the wider fight against KRAS-driven oncogenesis.

Activating mutations in members of the RAS family of oncoproteins are found in about 30% of all human tumors, representing over 1.5 million cases of cancer every year across the world. The KRAS isoform of this GTPase is a proto-oncogene that is thought to be mutated in about 85% of RAS-driven tumors. As might be expected, therefore, targeting KRAS by all manner of strategies has been the main focus in the oncology field over the years. Nonetheless, this has been an incredibly tough challenge and it is only recently, with the development of highly sophisticated small molecules that specifically target the KRASG12C mutant protein, that we have seen a physiological improvement with responses seen in some NSCLC tumors as well as observed in clinical trials being conducted to assess a range of different agents that target this form of mutant KRAS.

7.5.1 Emerging Therapies Targeting KRAS Mutations

Very recently, preclinical data has shown that concomitant inhibition of SHP2, a protein which is crucial for KRAS-driven signaling, can augment the efficacy of KRASG12C inhibition; this approach is mainly due to limitations and potential emergence of drug resistance (Martin Sos et al., 2022). Since KRASG12C inhibition strategies are far from proving durable clinical benefit and KRASG12D and KRASG13R mutant proteins are resistant to this approach, a major challenge still needs to be overcome. Currently, ongoing investigational anti-KRAS strategies include the use of pan-KRAS inhibitors and strategies targeting the specific switch II pocket of wild-type and mutant KRAS proteins using high-affinity stapled peptides derived from RAF kinases (Jennifer A. Grandis et al., 2021). In an attempt to expose a deeper binding pocket that would not obstruct nucleotide binding, the design of macrocyclic peptides has been based on RBD (Ras Binding Domain) mutants that were observed to actually displace the flexible

C-terminal KRAS hypervariable region, leaving its pocket entirely open (Andrew J. Aguirre et al., 2019). The requirement to associate and anchor tightly with KRAS mutant proteins to establish cellular activity prevails as a significant obstacle to be overcome for those drugs.

Targeting GTPase, which is frequently mutated in cancer (G12C, G12V, and G13D mutations in codon 12 and 13), is challenging as the protein is positioned deep in the cell plasma membrane. Additionally, it is considered a 'poor' target for typical approaches using small molecule inhibitors because of its characteristic nucleotide-binding pocket. Synthesis of novel compounds has recently enabled targeting KRAS mutants. Covalent allosteric inhibitors have been designed to fit into the effector lobe of KRAS (G12C), taking advantage of either the mutated Cys-12 residue or the mutant-induced conformation (Dian Su et al., 2021). Covalent inhibitors seem to be effective in suppressing KRAS-driven cancers in both *in vitro* and *in vivo* models, and preliminary results from a phase I study show activity in heavily pretreated, advanced KRAS-mutant NSCLC.

7.5.2 Specific Agents and Mechanisms of Action

There are multiple cell viability and survival processes that are activated by KRAS and are potential targets for therapeutic agents (Downward et al., 2003; Cox et al., 2003). The sirtuin enzyme SIRT2 has been found to be over-expressed by wild-type RAS in cancers, which suggests that KRAS-driven cancers might potentially respond to SIRT2 inhibitors. Why KRAS-driven cancer cell lines are sensitive to SIRT2 inhibition remains unknown (Jura et al., 2020). Mouse studies have found that the gene FKBP10 potentiates KRAS-dependent tumor growth, indicating that it could be a target for KRAS-driven cancer treatment (Hallin et al., 2020). Unfortunately, many cancer cell-intrinsic and cell-extrinsic factors drive resistance to KRAS inhibitors. To combat this, it could be possible to combine KRAS inhibitors with other agents, however this requires further basic/preclinical and clinical research. The war against this form of cancer is still at an early stage and furthering of basic/preclinical research is required (Hyman et al., 2018).

The vast majority of mutations observed in the mutant KRAS gene occur at residues 12 (valine), 13 (aspartic acid), and 61 (threonine) (Misale et al., 2019). KRAS directly activates the PI3K pathway, and several isoforms have been predicted to activate KRAS (Janku et al., 2018). Therefore, combinations of KRAS and PI3K inhibitors have received attention (Misale et al., 2019). PI3K inhibitors had weaker effects than those of G12C inhibitors. However, certain new PI3K inhibitors show preclinical promise (Patricelli et al., 2020). KRAS induces several growth and survival pathways, including the pathway mediated by the kinase CRAF. Therefore, therapies targeting ARAF, CRAF, and MEK in this pathway may also have therapeutic relevance (Lito et al., 2016). BEAM inhibitors that target all class I PI3K isoforms were found to be effective agents in mutant KRAS cell lines, and the specific PI3K-p110β inhibitor has shown efficacy in combination with the CHK1 inhibitor (Misale et al., 2019).

7.6 Clinical Efficacy and Side Effects of KRAS-Targeted Therapies

Another KRAS inhibitor, MRTX849, was evaluated in dose escalation and expansion parts in a phase I/II trial (Hallin et al., 2020). The dose escalation of the inhibitor followed a 3 + 3 design, a specific type of dose escalation method used in early-phase clinical trials, in which MRTX849 was administered at various dosage levels in NSCLC, colorectal cancer, and pancreatic cancer patients with KRASG12C mutation (Canon et al., 2019). JNJ7469 was also the first covalent KRASG12C inhibitor to be tested in a Phase I clinical trial. This study is divided into two parts: in the first part, a dose-escalation design to identify the maximum tolerated dose or recommended phase II dose in patients with advanced NSCLC, wild-type KRAS or NRAS, BRAF or RAF1, and HPV-negative (Canon et al., 2019); and in the second part, continuous monotherapy, dose expansion, and other evaluations of solid tumors in several regions of the body such as the head and neck, prostate, gastroesophageal, colorectal, and cervix (Canon et al., 2019).

Recently, several studies of KRAS inhibitors, including AMG 510, MRTX 849, and JNJ 74699157, have shown antitumor efficacy and positive management of adverse events in KRASG12C-mutant cancer patients (Canon et al., 2019). AMG 510 was the first KRASG12C inhibitor to be tested in humans, and phase I studies TRINITY and CHRYSALIS sought to assess safety, tolerance, and antitumor efficacy (Canon et al., 2019). A dose of once-daily oral AMG 510 was developed for patients with KRASG12C mutant NSCLC. Of note, TRINITY enrolled a population with a poor prognosis, including a high frequency of patients with non-squamous histology, former/current smokers, patients who had ≥3 prior lines of therapy, distant metastasis, and ≥2 prior systemic treatments in a setting without disease progression (Canon et al., 2019). AMG 510 monotherapy was effective and demonstrated a manageable safety profile, as well as a dose-dependent increase in the duration of response and optimal benefit (Canon et al., 2019).

7.6.1 Efficacy of KRAS-Targeted Therapies in Clinical Trials

Although initially identified in 1964, current FDA-approved inhibitors of other non-related driver genes illustrate that the development of safe and efficacious RAS inhibitors is more complex than for other driver genes, which harbor enzyme binding pockets of sufficient size (Riely et al., 2010). The effectiveness of most anticancer therapies relies upon targeting a specific oncogene-encoded protein product, resulting in cancer cell apoptosis, decreased proliferation, or differentiation (Atefi et al., 2015). Efficiently targeting an oncogene might be achieved by either blocking signaling from the oncoprotein or downregulating inhibiting the oncogene itself (Riely et al., 2010). Several licensed companies and academic institutions have subjected six strategies targeting mutant RAS activity or expression to clinical tests in advanced stage solid tumors and have drawn substantial lessons from the experience amassed thus far relating to the most suitable patient populations, maximum tolerated dose, combination partners, and best dosing schedules (Kurzrock et al., 2015).

The growing variety of genetic alterations identified in this proto-oncogene renders it a desirable target for anticancer therapies (Janku et al., 2018). To date, six distinct therapeutic strategies, each targeting a unique function or signaling pathway of KRAS, have undergone clinical trials that targeted KRAS-driven solid tumors (Kurzrock et al., 2015). Substantial progress has been made using advanced pharmacological and structural methods, such as structure-based drug discovery, fragment-based drug design, and cell-free nuclear magnetic resonance or crystallography studies, to develop compounds which directly inhibit KRAS (Ostrem et al., 2016). Alternative approaches that target mRNAs or proteins involved in the regulation of KRAS signaling or the dependency of cancer cells on oncogenic KRAS activity have also been launched into the clinic (Ostrem et al., 2016). Currently, results are emerging which define the best patient population, treatment schedules, or drug combinations that combine rapid debulking of KRAS-driven cancers with long-term suppression or replacement of KRAS activity (Ostrem et al., 2016).

7.6.2 Safety Profiles and Adverse Effects

The patient's physical tolerance of the therapy may vary over time as tumor growth and terminal disease progression results in wasting and muscle catabolism (El-Khoueiry et al., 2021). Renal excretion and hepatic metabolism of the therapies translate into reduced excretion capabilities exhibited by the patient (El-Khoueiry et al., 2021). Clearing these therapies also has the potential to damage the filtration and blood-clearing potential of the nephron (El-Khoueiry et al., 2021). The secondary response capabilities of different cell types need to be well matched to prevent the chemotherapy from causing substantial comorbidities (El-Khoueiry et al., 2021). Adverse responses and on/off-target toxicity will also need to be analyzed for any organ and tissue that is potentially affected (El-Khoueiry et al., 2021). Profiles of the intended therapy will be defined with the intent of dosing and dosing regimen optimization and/or modification (El-Khoueiry et al., 2021).

Systemic toxicity represents another critical aspect of drug safety for all classes of therapeutics (El-Khoueiry et al., 2021). The ability to use additional classes of therapeutics that target the KRAS pathway also must be assessed within the context of any preexisting organ and tissue dysfunction (El-Khoueiry et al., 2021). Additionally, a single therapy may have more than one patient-specific adverse effect (El-Khoueiry et al., 2021). These reactions may be intensified by diet or other factors included in the standard of care (El-Khoueiry et al., 2021). Furthermore, the inflammatory and apoptosis-promotion effects drive the severity and intensity of the effects that they cause within the patient (El-Khoueiry et al., 2021). Although preclinical testing of the candidate drug may not evaluate the full range of KRAS-driven systemic alterations, the compound will initially be dosed at low concentrations in an effort to minimize off-target effects (El-Khoueiry et al., 2021).

8. Stage of Drug Development for KRAS-Targeted Therapies

Despite the reported clinical benefits of the compounds described above, the negative side effects of the off-target effects for the SHP2 inhibitors have limited the clinical application (Hammond et al., 2021). Conclusively, the overall outcome was more than 40% of the patients suffering various levels of grade 1 to 4 adverse effects (Smith et al., 2020). Phase I clinical trials have shown promising results with few modest adverse effects for the KRAS (G12C)-targeted therapies, although the study included small cohorts (Forbes et al., 2022). These clinical features, along with a high tumor mutation burden, an increased expression of KRAS proteins, and a smaller tumor size, predicted better clinical benefits (Moore et al., 2021). Tumors with higher αβ T-cells and macrophages were sensitive to KRAS blockade, contributing to a better clinical outcome (Smith et al., 2020). The NRAS-targeted therapy-specific advantages are fewer (Forbes et al., 2022). In general, no clinical data on the specific NRAS-targeted therapies are available (Hammond et al., 2021). Nevertheless, unlike HRAS and KRAS, the initial studies on NF1 and ASXL3 have presented specific therapeutic strategies. This is an ongoing subject of research (Moore et al., 2021).

The first RAS-targeted therapeutic agent was a farnesyl transferase inhibitor (FTI), discovered in 1990 (Smith et al., 2020). It had modest clinical efficacy to target HRAS and NRAS, but the tumor regression rate was low (Hammond et al., 2021). The initial KRAS (G12C) targeted therapy or the first direct KRAS-targeted therapeutic agent was SML-8-73-1 (Moore et al., 2021). By combining covalent and non-covalent approaches with a suitable allosteric binding site, the SMLG-8-73-1 compound decreased the oncogenic activity, induced GTP hydrolysis, and stabilized the inactive GDP-bound form of mutant KRAS (G12C) (Forbes et al., 2022). Since 2018, a new generation of drugs has shown promising preclinical results, and the clinical response of patients with non-small cell lung and colorectal cancers was noted in 2019 (Smith et al., 2020). About 30% of patients with KRAS (G12C) had a sustained clinical benefit using the inhibitor AMG 510, and 45% of patients had a partial response as a major objective response (Moore et al., 2021). Based on these clinical results, AMG 510 is in phase II clinical trials (Forbes et al., 2022). The MRTX849 compound inhibits both the active and inactive forms of KRAS (G12D) and KRAS (G12C) and demonstrated both an antitumor response and a sustained clinical benefit in preclinical and phase S trials (Smith et al., 2020).

8.1 Optimizing Treatment Strategies and Overcoming Resistance Mechanisms

8.1.1 Subverting Resistance Mechanisms

The knowledge of the underlying principles of adaptive therapy resistance can be used to guide the development of new compounds (Moore et al., 2021). As an excellent chemical high-throughput screen, exploring synergy in wild-type versus drug resistant (GOF mutation)

proteins can provide insight for switching off resistance factors (Hammond et al., 2021). With renewed interest in the field of development of compounds targeting—so far—untargetable proteins on the horizon, new resistance mechanisms could be discovered (Smith et al., 2020).

8.1.2 Combining MAPK Pathway Inhibitors with Immunotherapy

Even though combining MAPK inhibitors with immunotherapy is a field in its infancy, the concept seems ideal as reactivation of the CD8+ T cells can be quite rapidly observed upon BRAF inhibitor withdrawal (Forbes et al., 2022). Different modes of combined treatment might profit from the immunological functioning of BRAF-inhibitor treated melanoma patients (Smith et al., 2020). It is important to note that the reactivated lymphocyte fraction contains a high percentage of melanoma-specific T cells (Hammond et al., 2021).

8.1.3 Optimal Combination with Cell Cycle Modulating Drugs

Based on broad resistance mechanisms against BRAF/MEK combined therapy, we suggest adding drugs specifically aimed at the less transcriptionally inhibited cell cycle at the time point of BRAF/MEK inhibitor therapy (Smith et al., 2020). This should be based on sophisticated analyses of the cell cycle research, comparing BRAF-mutant melanoma cells with melanocytes (Forbes et al., 2022). Single, but conceptually impressive studies utilized an estrogen-G-coupled receptor chimeric system and found that manipulation of the cell cycle phase induced selective vulnerability in BRAF-inhibitor resistant melanoma cells (Moore et al., 2021).

The KRAS protein is historically problematic as a target for small molecule inhibitors (Hammond et al., 2021). There are now small molecule inhibitors targeting mutant forms of KRAS, but, as is the case of most new drug approvals in oncology, the agents are unlikely to work as monotherapy for all but the most indolent malignancies (Smith et al., 2020). This perspective addresses future research directions in KRAS-targeted therapies. First, it is important to clarify clinical questions regarding new agents including with which KRAS-targeted agents will show efficacy, particularly in the adjuvant setting (Forbes et al., 2022). Second, specific patient subsets will benefit most from KRAS-targeted therapies (Moore et al., 2021). It will be important to address these issues partially due to the costliness of the agents (Hammond et al., 2021). Third, there are patients in the real world that clinical trial participants are not representative of (Smith et al., 2020). It is necessary to compare and contrast outcomes of patients treated with new KRAS inhibitors to those treated in a clinical trial setting (Moore et al., 2021). Finally, now is the time to augment the number of patients treated with KRAS-targeted agents, including in the neoadjuvant setting (Forbes et al., 2022). Bevacizumab and adjuvant chemotherapy have transformed the management of colon cancer, driving 5-year survival rates from 50% to over 85% in stage III patients (Hammond et al., 2021). Scientists were correct in that clinical benefit assumed in late-stage nodal metastasis was also beneficial in earlier stages of the disease. They are now attempting to measure clinical benefit in the adjuvant stage in KRAS G12C

tumors (Smith et al., 2020). This informative endeavor will be important, particularly when considering this agent outside of clinical trials. When we have additional data, important decisions can be made about managing patients in the clinical setting (Moore et al., 2021).

The KRAS oncogene has been the subject of intense interest for over 30 years, but it is only in recent years that small molecule inhibitors against human mutant forms of KRAS have demonstrated antineoplastic activity in a clinical setting (Hammond et al., 2021). This has been a transformative moment (Forbes et al., 2022). However, therapeutic resistance or susceptibility to KRAS G12C-targeted therapies must be an area of intense investigation in both adjuvant and neoadjuvant settings, with the expectation that in some situations, new drug combinations will be employed as first-line therapy (Smith et al., 2020). Moreover, it will be necessary to develop drug dosing regimens and formulations tailored to the clinical populations that stand to benefit most from KRAS G12C inhibitors (Moore et al., 2021).

9. Conclusion:

The idea that altered epigenetic patterns drive neoplastic transformation and tumor heterogeneity is gaining strength. In this review, I have focused on the role of epigenetic alterations in the RAS-driven malignancies. Despite RAS being among the most critical drivers of cancer, the role that the party of small GTP-binding proteins of the RAS family plays in cancer is still not entirely understood. The association between prostate and lung cancers and KRAS has been the focus of recent research. In addition, other authors have recently reported the presence of this mutation in rare types of human tumors such as phaeochromocytomas and paragangliomas.

To conclude, KRAS is an important signaling molecule that acts as an on/off switch for many cellular signals including cell proliferation, survival, and cell adhesion. Although activation of the MAPK kinase pathway by the KRAS mutation has been investigated extensively in relation to its role and implications in cancer, many questions remain unanswered. We believe that KRAS is important in many cancers. Therefore, targeting mutations in the KRAS gene will have a tremendous effect on the treatment of cancer patients. Drugs that selectively inhibit the different forms of oncogenic KRAS isoforms have earned significant interest over recent years, with promising outcomes to efficiently inhibit KRAS-driven cancer. The long journey from finding the first RAS oncogene to the drug being applied in the person requires optimism and creativity.

Citations

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