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**Master thesis**

# The Incidence of KRAS G12D in Colon Cancer

# Η Επίπτωση του KRAS G12D στον Καρκίνο Παχέος Εντέρου

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**Περίληψη**

Ο καρκίνος παχέος εντέρου (ΚΠΕ) αποτελεί τον τρίτο συχνότερο καρκίνο στους άνδρες και τον δεύτερο στις γυναίκες παγκοσμίως. Μεταλλάξεις στο γονίδιο KRAS προκύπτουν σε ποσοστό 40-52% των περιπτώσεων ΚΠΕ οι οποίες επίσης παρατηρούνται στο ένα τέταρτο όλων των καρκίνων του ανθρώπου συνολικά. Στην παρούσα εργασία παρουσιάζεται ο φυσιολογικός ρόλος της πρωτεΐνης RAS και ο καταρράκτης αντιδράσεων που προκύπτει από την ενεργοποίησή της. Η ανάλυση του σηματοδοτικού μονοπατιού δίνει μία ματιά εκ των έσω στις δράσεις της πρωτεΐνης, βελτιστοποιώντας έτσι τη δυνατότητα δημιουργίας αναστολέων καρκινογένεσης. Στους επιτόπους 12, 13, 61 και 146 του γονιδίου έχουν αναγνωριστεί πολλαπλές μεταλλάξεις και έχουν ενοχοποιηθεί για το κακόηθες δυναμικό τους. Συγκεκριμένες αντικαταστάσεις όπως η G12D αποτελούν στόχο αντικαρκινικών θεραπειών. Επιπλέον, η ανάλυση των μηχανισμών που προκαλούν ενδοκυττάρια διακίνηση πρωτεϊνών απέδωσε έναν εναλλακτικό τρόπο στόχευσης καρκινικών μορίων. Η KRAS πρωτεΐνη θεωρείται ένας ισχυρός προγνωστικός και προβλεπτικός δείκτης ανταπόκρισης στην θεραπεία με αναστολείς EGFR. Πολυάριθμες κλινικές μελέτες με ενώσεις που εμποδίζουν την ενεργοποίηση της πρωτεΐνης ή μπλοκάρουν ένα από τα τρία βασικά σηματοδοτικά μονοπάτια του καταρράκτη KRAS ή τέλος, την αλληλεπίδρασή της με την κυτταροπλασματική μεμβράνη είναι σε εξέλιξη.

**Abstract**

Colorectal cancer (CRC) is the third most commonly occurring cancer in men and the second most commonly occurring cancer in women. Mutations in the KRAS gene occur in 40–52% of CRC cases which are also noted in nearly one-quarter of a wide spectrum of cancers. Herein is presented the physiological role of the RAS protein and the detailed downstream cascade after its activation. The analysis of this signalling pathway gives an insight into its actions, thus facilitating the development of inhibitors to block tumorigenesis. There are multiple mutations in residues 12, 13, 61, and 146 of this gene and they play a critical role in tumorigenesis. Specific substitutions such as the G12D are targets of antitumor therapies. Furthermore, the identification of the mechanisms that cause intracellular protein trafficking has suggested several potential therapeutic approaches. KRAS is an important prognostic factor and a predictive marker in determining resistance to epidermal growth factor receptor (EGFR) inhibitors. Here are several ongoing clinical trials of compounds that impede either the activation or the protein-membrane interaction or one of the three main signalling pathways of the KRAS cascade.

**Keywords:** KRAS, G12D, mutation, colorectal, cancer, trials, therapy

## Introduction

In this study, we review the physiological role of the KRAS protein and its oncogenic dynamic when mutations occur. Point mutations that alter amino acids G12, G13, Q61, or 146, lead to a loss of the intrinsic GTPase activity. Herein we investigate specifically the G12D mutation which arises from a single nucleotide substitution of the glycine (G) at position 12 by an aspartic acid (D) and its incidence in CRC. The current understanding of this protein's actions has offered an inside view on a molecular basis which allows the creation of inhibitors that will impede the oncogenic activity. At present, there are multiple clinical trials with components that target a specific fold of the KRAS cascade.

## Materials and methods

Information from the database of clinicaltrials.gov for the ongoing trials was collected and analysed. A review of the literature was conducted with the keywords KRAS, G12D, Colorectal Cancer, and Targeted Therapy were used to search the journals and websites Pubmed, ScienceDirect, Nature, American Journal of Cancer Research, Cell, Cancer Research, Clinicaltrials.gov, COSMIC and Protein Bank Database to collect data for the mutations that occur in the KRAS oncogene, their frequency, their proportions in every human cancer, the structure and the physiological function of the KRAS protein and more specifically the KRAS G12D mutation, its impact in CRC and the trials that focus in targeted treatments against this oncogene.

## Results

The RAS oncogene has a physiological role in human cells. It is the beginning of signalling transduction resulting in cell proliferation, tumoural overgrowth, and inhibition of apoptosis. This signalling is illustrated with a 3-way downstream cascade starting from the cell membrane and ending at the nucleus. Nonetheless, specific mutations occur, causing functional changes in the protein, thus leading to the oncogenic transformation of the cells. Several ongoing clinical trials show promising results in the blockage of this oncogenic cascade of the mutated cells which carry the mutation G12D in CRC.

### 1. The RAS family

The RAS family consists of the proto-oncogenes Kras, Hras, and Nras and their proteins. Historically, KRAS (Kirsten rat sarcoma viral oncogene) is a proto-oncogene that was first described by Werner H. Kirsten in 1967, after the discovery of a murine sarcoma virus during his extensive study of acutely transforming retroviruses isolated from mice, rats, cats, monkeys, chickens, and turkeys. This oncogenic virus causes rapid formation of sarcomas in infected

animals and potently transforms cells in culture. In 1982, Weinberg and Barbacid isolated a gene from human bladder cancer cell lines. Subsequently, this gene was identified as a homolog of the RAS gene and was named HRAS. It was located on the short arm of chromosome 11 (11p15.1–11p15.3)12. In the same year, another homologue, called KRAS, was found in human lung cancer cells and this was located on the short arm of chromosome 12 (12p11.1–12p12.1). The last gene of the family was found in human neuroblastoma, it is called NRAS, and is located on the short arm of chromosome 1 (1p22–1p32).

The structural similarities in members of the RAS family proteins and genes are evolutionarily conserved from lower animal species like yeast to humans. This supports the ubiquitous nature of RAS proteins and suggests the importance of RAS proteins in the growth and development from lowest to highest eukaryotes species.

The RAS genes are composed of 4 coding exons and 1 non-coding exon distributed on the full length of approximately 30 kb DNA. The KRAS gene encodes two highly related protein isoforms, KRAS-4B and KRAS-4A. The term KRAS is generally referred to as KRAS-4B due to

the high level of mRNA encoding KRAS-4B in cells. The translation product is a guanine nucleotide-binding protein (GTPase), consisting of amino acids and polypeptides with 21 kD mass and thus KRAS is also known as the p21 gene. Genetic alterations, in which a single base pair substitution alters the genetic code, are the main cause for the activation of these proto-oncogenes into oncogenes.[1]

**Fig. 1.1:** The frequency of isoforms HRAS, NRAS, and KRAS mutations in human cancers[1]

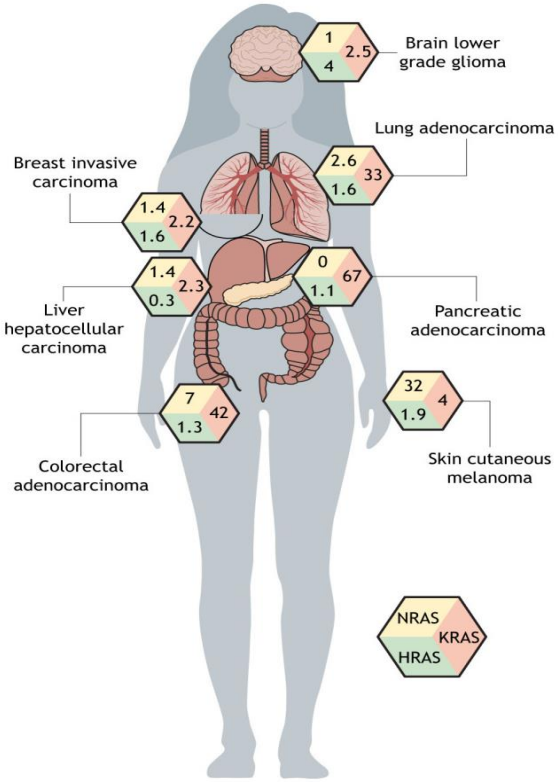


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Fig. 1.1 demonstrates the frequency of each RAS isoform (HRAS, NRAS, KRAS) mutations in human cancers and the proportion of human tumours with mutations in each RAS gene.

RAS genes are mutated in close to a third of all human cancers and in many tumour types they represent the main oncogenic driver. Mutations in *KRAS* have been reported in 60–90% of pancreatic cancer cases whereas in colorectal and lung cancers, they are found in 30–50% of tumours and NRAS is mutated in ~30% of melanomas[2]**.** The HRAS gene is most frequently mutated in brain cancers, such as low-grade glioma.

## 1.1 The structure of KRAS protein

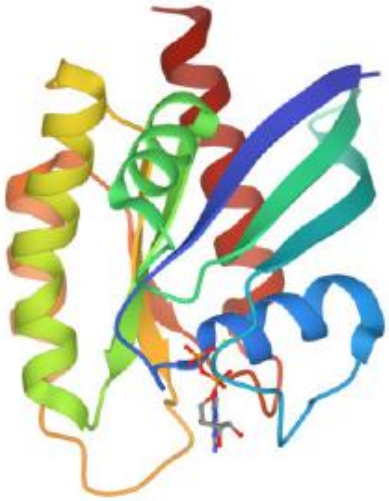

**Fig. 1.2**: Crystal structure of KRAS, demonstrated by x-ray crystallography. 5 α-helices and 6 β-sheets with a GTP molecule bound in the G-domain (https://www.rcsb.org/search)

The structural appearance of the protein (Fig.1.2) is crucial knowledge for the development of molecules and enzymes which will connect covalent or noncovalent to the protein and its effector domains. Since the discovery of X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, our understanding of the protein's body and its conformational changes has expanded. This technique has helped researchers to visualise the protein in 3D pictures and process its details (Figure 1.2).

The crystal structure of the RAS protein reveals 189 amino acids which are organised in 6 β-strands and 5 α-helices which form two major domains: a catalytic domain called the G domain (effector loop) and a hypervariable region (HVR). Furthermore, the G domain consists of three regions: Switch I (amino acids 30–38), Switch II (amino acids 59–67), and the P loop, which binds guanine nucleotides. The G domain is furthermore attached to the C-terminal HVR which comprises the CAAX motif. The latter is responsible for membrane localization and attachment.

Specifically, the HVR domain terminates with a CAAX motif, where C is cysteine, A is any aliphatic amino acid and X is any amide acid, which acquires lipids by farnesyl or prenyl modification. Cysteine is prenylated by farnesyltransferase (FTase) and the −AAX portion left after prenylation is removed by RAS-converting enzyme 1 (Rce1)[5].

Isoprenylcysteine-carboxyl-methyltransferase (ICMT) then methylates the carboxyl group of the newly exposed isoprenylcysteine. Lipid modifications formed by this reaction provide weak membrane binding affinity which is stabilised by a second signal motif. These modifications help the localization of the protein in the cellular membrane. This interaction is predominantly stabilised through two basic residues R161 and R164 in the HVR in association with the adjacent membrane anchor[6].

The 4 isoforms of the RAS proteins, namely KRAS 4A, 4B, NRAS, and HRAS are highly conserved (~90%) throughout the amino-terminal GTPase domain (amino acids 1–166), which contains the GTP–GDP binding site and interaction sites for effector proteins, but differ in the carboxy-terminal portion, termed the hypervariable region (Figure 1.3). Ras family members share substantial primary sequence homology in their N termini, particularly in the phosphate-binding loop (P-loop) and the nucleotide-sensitive switch I and II regions. The C terminus contains the membrane-targeting CAAX sequences

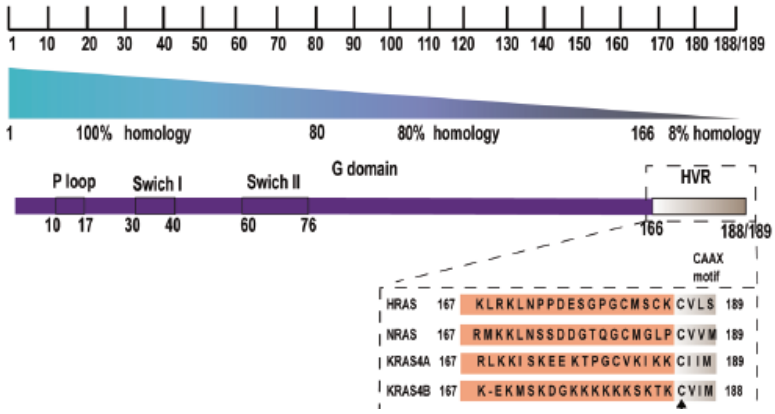


**Fig. 1.3**: The GTP domain is shown to be the same in the 4 RAS proteins, the different HVR regions, and the CAAX motif[3]

### 1.2 Physiological functions of KRAS

As mentioned before the KRAS is a guanine nucleotide-binding protein that has an intrinsic GTPase potentiality. It appears in the cell in two forms. In the inactive state, it binds GDP, and in the active state the GTP. This G-protein operates like a binary switch, using a GTP–GDP–GTP cycle to flip back and forth between an on and an off state. Key regulators between these two modes are guanine nucleotide exchange factors (GEFs), which activate KRAS by promoting the replacement of GDP by GTP, and GTPase activating proteins (GAPs), which trigger its GTPase

activity, thereby inactivating it (Fig. 1.4). An example of GEF is the protein Son Of Sevenless (SOS) which has been a therapeutic target in some clinical trials, with promising results.

Ras was found (1) to bind a GDP molecule when in its inactive state; (2) to jettison its bound GDP after receiving some stimulatory signal from upstream in a signalling cascade; (3) to acquire a GTP molecule in place of the recently evicted GDP; (4) to shift into an activated, signal-emitting configuration while binding this GTP; and (5) to cleave this GTP after a short period by using its intrinsic GTPase function, thereby placing itself, once again, in its non-signal-emitting configuration (Fig. 1.5).

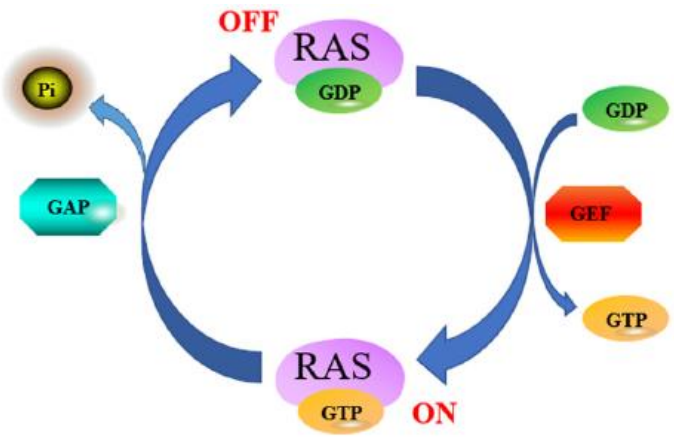


**Fig. 1.4**: KRAS activity regulation by GEF and GAP[4]

In effect, the Ras molecule seemed to behave like a light switch that automatically turns itself off after a certain predetermined time.

The active GTP-bound form enables a cascade of reactions that regulate a downstream pathway of signalling. This chain reaction is mediated by multiple phosphorylations of kinases that transduce a message. This pathway also interferes with upstream regulators with negative feedback. Ultimately, the receivers of this signalling are specific transcription factors (TFs) that trigger the activation of genes that are anchored in the nucleus and are involved in the cellular proliferation, differentiation, and inhibition of apoptosis.

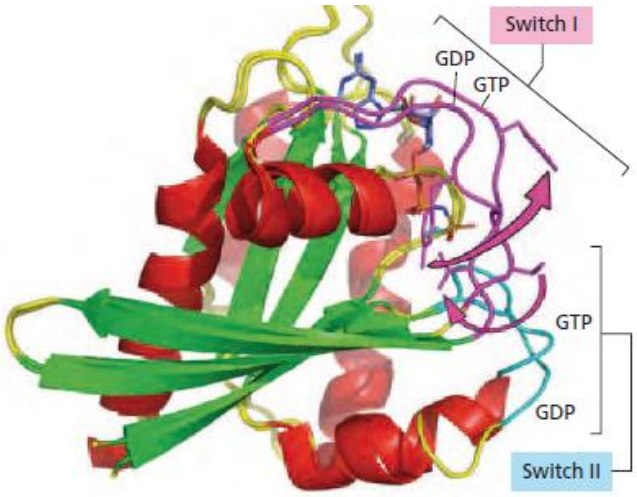


**Fig. 1.5:** The replacement of GDP by GTP, which results in the activation of Ras signalling, also causes a shift in two regions of the Ras protein (depicted here from a slightly different perspective). Both Switch I (magenta) and Switch II (aquamarine) regions undergo the GDP-to-GTP conformational shift (as indicated by the positions of GDP and GTP). The conformational shift for Switch I is also indicated by the magenta arrows. These shifts allow the Ras protein to interact physically with its downstream effectors. The guanine nucleotide is indicated by a stick figure[4]

### 1.3 The role of KRAS

The role of KRAS stands in the middle of a complex signal-transducing cascade. The original stimulators are extracellular signals which bind with ligands on the cell surface and regulate tyrosine kinase receptors. There is a linear connection between the ligands and the RAS protein, but as soon as the GDP compound is dephosphorylated by a GEF and gives space for the GTP to board on the G-domain of RAS, then there are 3 mainly different pathways with 3 different downstream targets in the nucleus who will receive the initial message (Fig. 1.6).

A basic and common way of signal transduction is through the phosphorylation of proteins mediated by Tyrosine Kinases (TKs). The receptor-associated tyrosine kinase phosphorylates a series of target proteins in the cytoplasm. Such covalent modification alters the three-dimensional conformation and the stereochemistry of the proteins, thereby placing them in an active signalling

state that furthermore allows them to transfer signals to a partner one step further down in the signalling cascade. This model explains efficient communication between cellular molecules.

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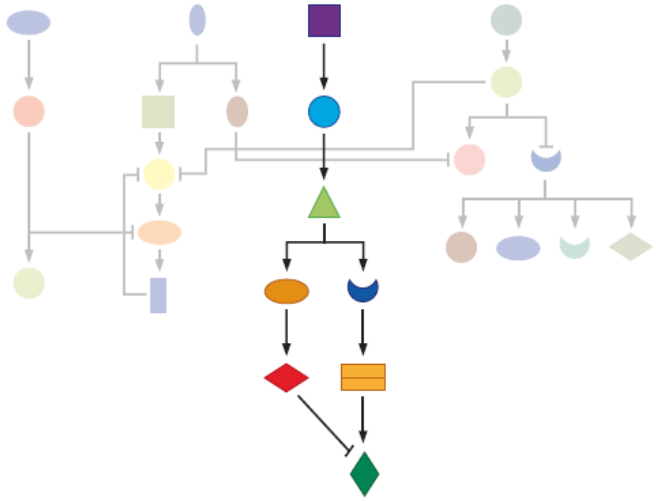
**Fig. 1.6**: This diagram of imaginary signalling circuitry illustrates how a signal transduction



cascade (a series of signalling proteins that operates much like a molecular bucket brigade) passes signals from an upstream source (purple square) to its intended downstream target (dark green diamond) and, at the same time, avoids inadvertent activation of dozens of other signalling proteins in the cell (faintly drawn symbols). Arrowheads denote a stimulatory signal, while a line at right angles (a crossbar) denotes an inhibitory signal[4]

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In mammalian cells, there have been recognized and analysed over 100 different kinases. About 50 of them selectively phosphorylate tyrosine residues in different substrates and they are called tyrosine kinases.

There are many serine/threonine kinases. Their actions are converted by phosphatases. These are enzymes that use water to cleave a phosphoric acid monoester into a phosphate ion and an alcohol. Because a phosphatase enzyme catalyses the hydrolysis of its substrate, it is a subcategory of hydrolases.

As mentioned before KRAS is a GTPase protein, but GDP to GTP conversion is an intrinsic effect of KRAS. How does the protein interact with other molecules? How does it represent an effective signal transductor? When Ras binds GTP, two of its “switch domains” shift, enabling its effector

loop to interact physically with several alternative downstream signalling partners that are known collectively as Ras effectors, that is, the proteins that carry out the actual work of Ras. Each of these effectors binds quite tightly to the effector loop of the GTP-bound form of Ras protein while having a little affinity for the loop presented by its GDP-bound form.

The effector loop interacts with at least three important downstream effectors of Ras. Amino acid substitutions affecting three residues in the effector loop create three alternative forms of the Ras protein that interact preferentially with either PI3 kinase, Raf, or Ral-GEF.

### 1.4 Knock-Knock: Who's there? The initial stimulus

The initial stimulus of the signalling pathway is an outer cellular message which is received by receptors anchored in the cell surface. These ligands gather a variety of signals and their role is to funnel them into the cytoplasm. The basic components of this process are growth factors like Endothelial Growth Factor (EGF) and platelet-derived growth factor-β (PDGF-β). Once they are bound in their Tyrosine Kinase Receptors (RTKs) the downstream cascade begins.

RAS proteins primarily reside at the cytosolic side of the plasma membrane by way of tethering specific lipid moieties added to the carboxyl termini. Upon activation of an RTK, for example, epidermal growth factor receptor (EGFR), the growth factor receptor-bound protein 2 (GRB2), a 'bridging' protein that contains two SH3 groups and one SH2 group, interferes between the EGFR and a protein called Son-Of-Sevenless (SOS) which acts as a GEF. Its SH3 domains have an affinity for two distinct proline-rich sequences present in Sos, while its SH2 sequence is associated with a phosphotyrosine present on the C-terminus of many growth factors–activated receptors. Consequently, SOS, which seems usually to float freely in the cytoplasm, now becomes tethered via the Grb2 linker to the receptor. These bridging-adaptor proteins are the Grb2 and the Shc. They both have been targeted with molecules to reduce their activity in many anti-cancer trials.

In summary, the initial stimulus reaches the cell surface (TK-RTK) and enables the building of a bridge (Grb2/Shc-GEF) to transfer the message to the RAS guanine nucleotide exchange factors (GEFs). The latter is recruited to the plasma membrane and exchange of GDP for GTP on RAS, generating an active GTP-bound RAS. Immediately after that RAS GTPase-activating proteins (GAPs) accelerate the GTPase activity of RAS, returning RAS to the inactive GDP-bound state. Thus, localization to the cell membrane is required for KRAS GTP binding and activation (Fig. 1.7).

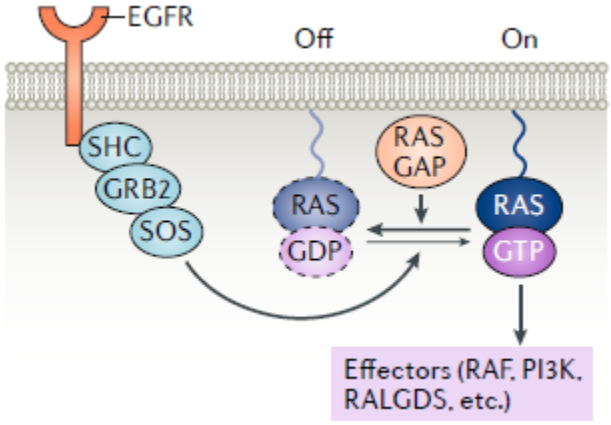


**Fig. 1.7**: EGFR binding to its receptor, the Shc-Grb2-SOS bridge, and the localization and activation of RAS protein to the cell membrane[5]

Post-translational modification of KRAS is the first step of membrane localization, and there are three key enzymes involved in this process: farnesyltransferase (FTase) or geranylgeranyltransferase (GGTase), RAS-converting enzyme (RCE1) and isoprenylcysteine carboxyl methyltransferase (ICMT). This interaction plays a key role in the activation of the RAS protein and it has reached the attention of many studies. Currently, multiple preclinical trials target this specific activity.

### 1.5 RAS-regulated signalling pathways: A cascade of kinases

Once the initial stimulus is bound to the cell membrane and transferred to the RAS protein via the adaptor bridge, the RAS protein is active. The multiple cellular responses that a growth factor elicits can be explained by the fact that its cognate receptor, which specifically binds it, can activate a specific combination of downstream signalling pathways. Each of these pathways might be responsible for inducing, in turn, a biological change that occurs after the cell is stimulated by this particular growth factor. Moreover, exaggerated forms of this signalling are operating in cancer cells that experience continuous growth factor stimulation because of an autocrine signalling loop or a mutant-activated receptor.

There are three main downstream signalling cascades that radiate from the RAS activation: Ras-Raf-MEK-ERK 1/2, Ras-PI3K-PIP3-Akt/PKB, and Ras-RAL-GEF. Each one of these pathways

transduces the initial stimulus from the cell surface to the nucleus. The main bearers of this message are kinases that can phosphorylate other proteins or other kinases and activate them. Through these chain reactions, the primary message arrives at the nucleus, enables transcription factors, evokes proliferation, and promotes cell survival, migration, DNA synthesis, and angiogenesis.

### 1.5.1 The Ras → Raf → MEK → Erk pathway

As mentioned before, when Ras binds GTP, two of its “switch domains” shift, enabling its effector loop to interact physically with several alternative downstream signalling partners that are known collectively as Ras effectors, that is proteins that carry out the actual work of Ras. One of these effectors is the Raf kinase which can phosphorylate substrate proteins on their serine and threonine residues. Raf is the acronym for rapidly accelerated fibrosarcoma. So once Ras has bound GTP, its affinity for Raf increases by three orders of magnitude, enabling the relatively tightly binding to Raf and doing so via the Ras effector loop. Raf is found in the cytosol before this association occurs. Thereafter, the protein becomes tethered via Ras to the plasma membrane.

Ras-GTP leads to the activation of the Raf kinase family (A-, B-, and C-Raf) by a multistage process that involves homodimer and heterodimer formation. More precisely, activated Ras accelerates the dimerization and phosphorylation of its downstream RAF proteins. This process has also the potential to cause a cascade of amplification. The reason is that one protein kinase can catalyse the phosphorylation of many substrate molecules. If the substrate is a protein kinase, it too can catalyse the phosphorylation of many substrate molecules, etc[6]. In this cascade there are feed-forward and feedback mechanisms. It is also not a linear and unidirectional signalling pathway, as it has multiple inputs and outputs and multiple scaffold proteins that dynamically regulate signalling. This is a basic characteristic of the dynamic of this signalling pathway and explains why it is so difficult to inhibit.

Raf now acquires active signalling powers and proceeds to phosphorylate and thereby activate a second kinase known as MEK which is also a serine/threonine kinase. This protein is also called mitogen-activated protein kinase and is commonly known as MAP2K, MEK, and MAPKK. The acronym MEK derives from MAPK/ERK Kinase. Afterward, MEK uses its power to phosphorylate two other kinases, the extracellular signal–regulated kinases 1 and 2, commonly referred to as Erk1 and Erk2. These are considered mitogen-activated protein kinase MAPKs. Generically, a kinase responsible for phosphorylating a MAPK is termed MAPKK; in the case studied here, this role is played by MEK. So MEK is a MAPKK and the kinase responsible for phosphorylating a

MAPKK is therefore classified as MAPKKK. In this case, RAF is the MAPKKK of the cascade (Fig. 1.8).

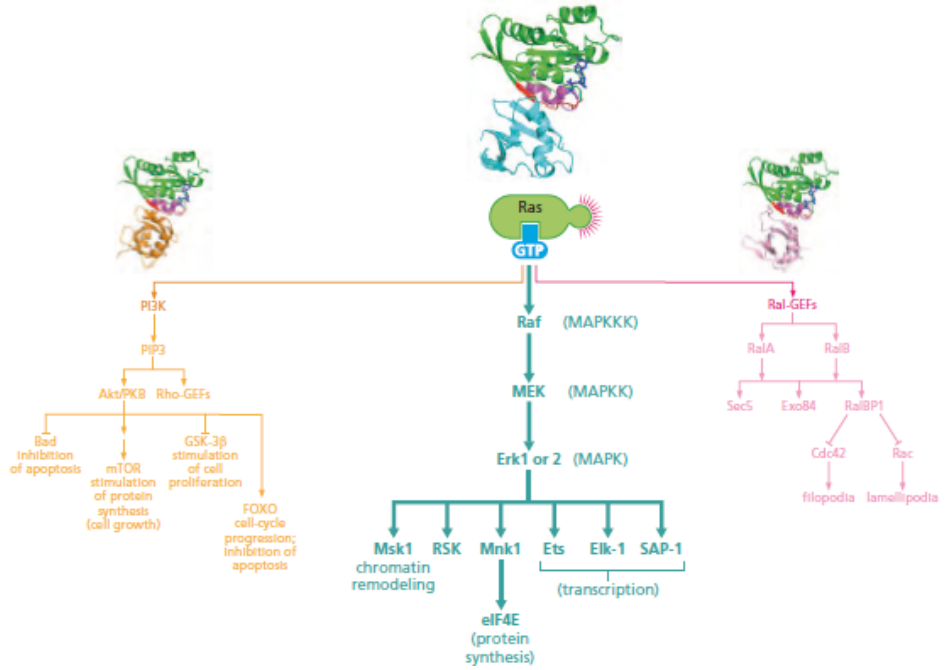


**Fig. 1.8**: The RAS-RAF-MEK-ERK1/2 cascade. The downstream actions of kinases start when Ras is activated by binding GDP. This results in cell proliferation and protein synthesis[4]

Once activated, an Erk kinase at the bottom of this cascade translocates to the nucleus, where it causes the phosphorylation of transcription factors; some of the latter then initiate the immediate and delayed early gene responses. Several transcription factors such as Ets, Elk-1, and SAP-1 phosphorylate and thereby activate other kinases, which proceed to the activation of yet other transcription factors. At the end of this downstream two chromatin-associated proteins HMG-14 and histone H3 are phosphorylated and this modification places the chromatin in a configuration that is more hospitable to transcription. The Mnk1 kinase is a cytoplasmic substrate of Erk1 and Erk2 and can phosphorylate the translation initiation factor eIF4E, thereby activating the cellular process responsible for protein synthesis.

It is important to notice that at the end of this downstream two immediate early genes are activated which encode the Fos and Jun transcription factors. Once synthesised, these two proteins can associate with one another to form AP-1, a widely acting heterodimeric transcription factor that is

often found in the hyperactivated form in cancer cells. These reactions are the hallmarks of tumorigenesis because they connect two basic cellular changes: the consistent activation of Ras protein, which derives from a mutation of the KRAS gene, such as the G12D, and excessive cell proliferation.

### 1.5.2 The Ras → PI3K → PIP3 → Akt/PKB → mTOR pathway

A second important downstream pathway of the KRAS protein evokes yet other cellular responses. In the context of cancer, the most important of these is the suppression of apoptosis. This anti-apoptotic effect is especially critical for cancer cells since many of them have a deviated cell suicide program. This is catalysed by the Phosphatidylinositol 3-kinase (PI3K) which is one of the three main effector pathways of RAS (Fig. 1.9). Cell growth, survival, cell cycle, cytoskeleton reorganisation, and metabolism are regulated by this pathway.[7]

The phospholipids of the cell membrane serve simply as a barrier between the aqueous exterior and interior environments. Their amphipathic structure consists of a hydrophilic head, which likes to be immersed in water, and a hydrophobic tail, which prefers non-aqueous environments. Because of this polarity of lipid bilayers such as the plasma membrane, the hydrophilic groups face and protrude into the extracellular and cytosolic aqueous environments while the hydrophobic tails are buried in the middle of the membrane.

Some phospholipids contain, at their hydrophilic heads, an inositol group which is a water-soluble carbohydrate molecule. The inositol moiety of such phospholipids can be modified by the addition of phosphate groups and the resulting phosphoinositol can then be cleaved from the remaining hydrophobic phospholipid molecule. Since it is hydrophilic it can diffuse into the cytoplasm and act as an intracellular hormone to dispatch signals from the plasma membrane to distant parts of the cell. Such intracellular hormones are often called *second messengers*. Alternatively, a phosphorylated inositol can remain attached to the phospholipid and thus can remain embedded in the plasma membrane where it can serve as an anchoring point to which certain proteins can become attached.

Phosphatidylinositol 3-kinase (PI3K) is a kinase that shows specificity for phosphorylating the 3' hydroxyl of the inositol moiety of membrane-embedded phosphatidylinositol (PI). While several distinct PI3 kinases have been discovered, the most important of these is the PI3K that phosphorylates the PIP2. PIP2 is a phosphatidylinositol (PI) that has phosphates attached to the 4' and 5' hydroxyl groups of inositol. It receives a modification from the PI3 and inositol acquires

yet another phosphate group, thus converting PIP2 into PI(3,4,5)P3, that is, PIP3. So PI3K attaches phosphate to a phospholipid.

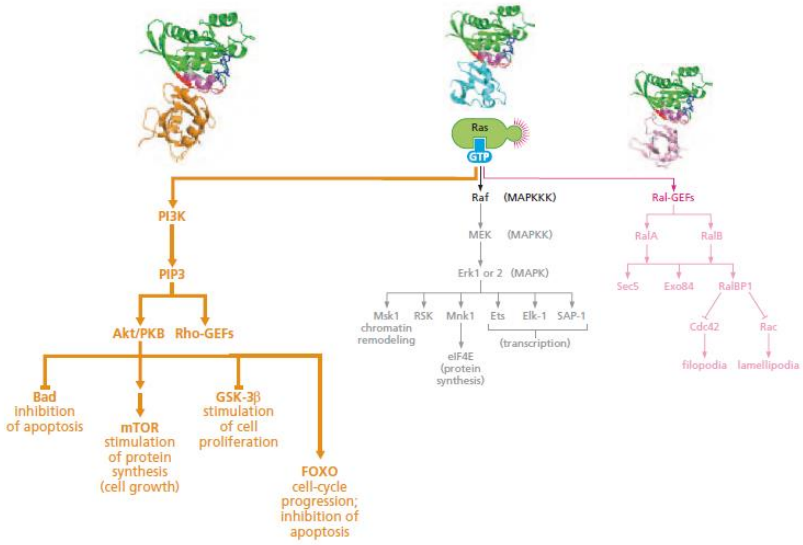


**Fig. 1.9**: The association between Ras activation and PI3K results in the formation of PIP3. This leads in turn to the tethering of Akt/PKB) and Rho guanine nucleotide exchange proteins (Rho-GEFs), to the plasma membrane[4]

The HRAS protein can also, effectively, activate PI3K only when the p85 regulatory subunit of PI3K is bound to phosphotyrosine on a ligand-activated growth factor receptor. Hence, PI3K functions as a direct downstream effector of Ras. When PI3K binds to Ras, this causes PI3K to become closely associated with the plasma membrane, where its PI substrates are located.

Akt, a serine/threonine kinase, also known as protein kinase B (Akt/PKB) has a strong affinity for this triply phosphorylated inositol head group. Thus, once PIP3 is formed by PI3K, an Akt/PKB kinase molecule can become tethered to the inositol head group of PIP3 that protrudes from the plasma membrane into the cytosol. Once activated, Akt/PKB proceeds to phosphorylate a series of proteins that have multiple effects on the cell, including enhancing cell survival by deactivating the cell apoptotic suicide program, stimulating cell proliferation and growth, and increasing protein synthesis. In addition, it also exerts an influence on cell motility and angiogenesis producing new blood vessels.

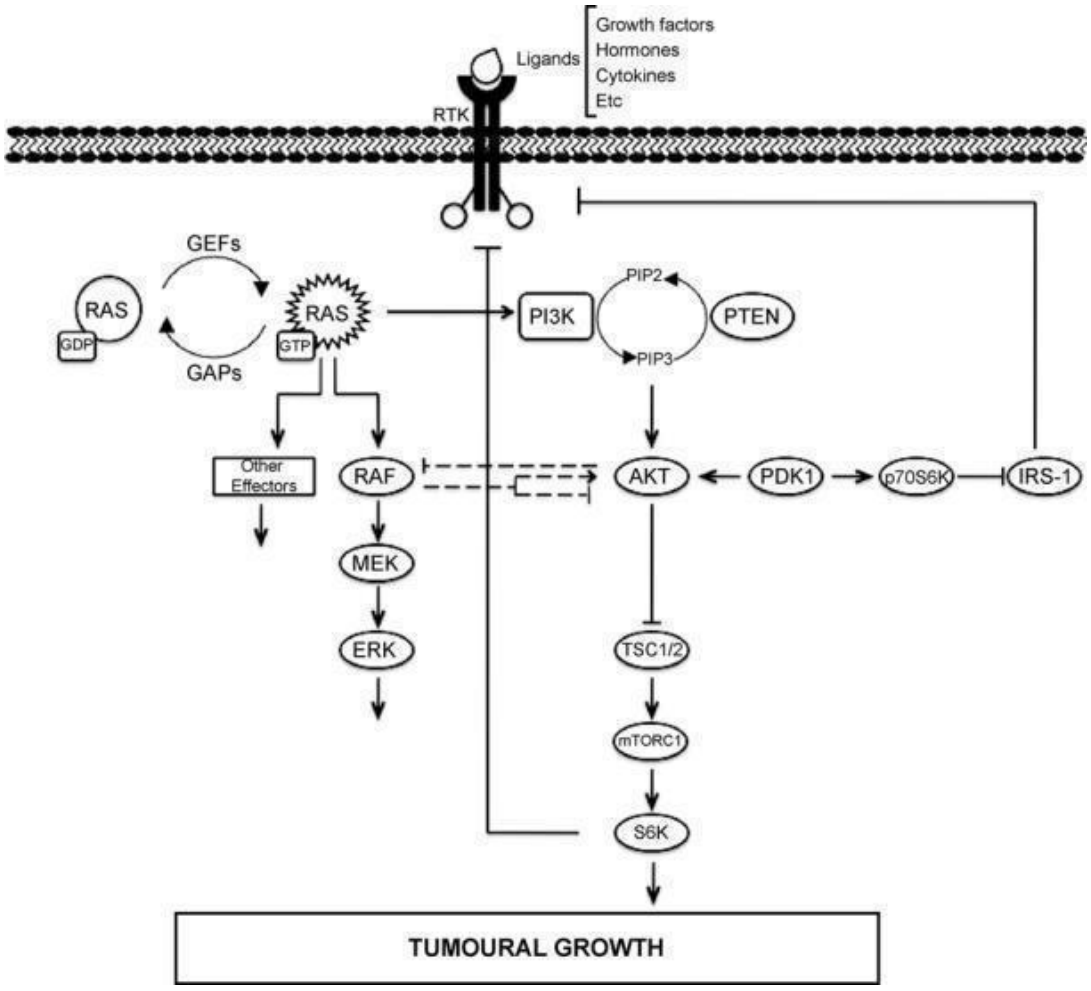


**Fig. 1.10**: Crosstalk and feedback in ERK and PI3K signalling downstream of RAS[8]

On the other hand, PTEN, a phosphatase that is been encoded by the homonymous housekeeping tumour-suppressing gene, can remove the 3' phosphate group from PIP3 that has previously been attached by PI3K (Fig. 1.10). Thus, acting on the opposite side of PI3K. This removal suggests two distinct mechanisms by which the Akt/ PKB signalling pathway can become deregulated in cancer cells—hyperactivity of PI3K or inactivity of PTEN. These signalling components are two of the most frequently mutated proteins in human cancers, resulting in dysregulated activation of PI3K signalling and providing undisputable genetic evidence of the central role of this pathway in tumorigenesis.[8]

In conclusion, PIP3 is a lipid second messenger that activates many downstream molecules. Another principal target of this messenger is the protein serine/threonine kinase AKT, also known

as PKB. The binding of PIP3 to AKT leads to the membrane recruitment of AKT and subsequent phosphorylation by the mTOR (mammalian target of rapamycin). This leads to the full activation of AKT, which in turn phosphorylates many target proteins, thereby regulating a range of cellular functions. An important target of AKT is the forkhead (FOXO) family of transcription factors. The main biological functions of these signalling pathways are cell metabolism, cell cycle and survival, protein synthesis, cell polarity and motility, and vesicle sorting.[9]

At the end of this second pathway appear the results of the activation of AKT/PKB. One of these is the inhibition of several proteins that play a prominent role in favouring the entrance of a cell into apoptosis (Fig. 1.11). Moreover, it induces dramatic changes in the proteins that control the rate of protein synthesis in the cell. Akt/PKB triggers the activation of mTOR (mammalian target of rapamycin) kinase; the latter can phosphorylate and inactivate 4E-BP, a potent inhibitor of translation, and, at the same time, activates p70S6 kinase, an activator of translation, by phosphorylation.

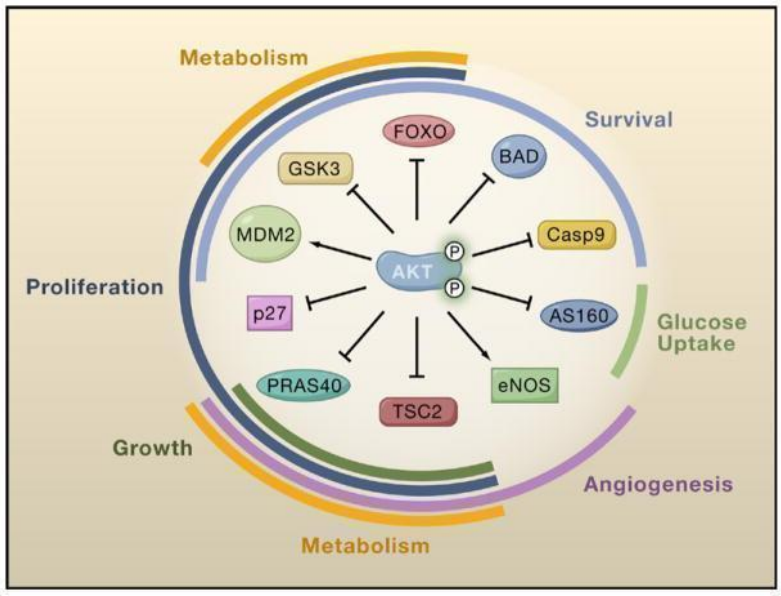


**Fig. 1.11**: Cellular functions of ten Akt substrates: Akt-mediated phosphorylation of these proteins leads to their activation (arrows) or inhibition (blocking arrows). Regulation of these substrates by Akt contributes to the activation of the various cellular processes shown (i.e., survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis). As illustrated by these ten targets, a high degree of functional versatility and overlap exists among Akt substrates[10]

These changes increase the efficiency with which the translation of a class of mRNAs is initiated; the resulting elevated rate of protein synthesis favours the accumulation of many cellular proteins and is displayed in the growth, rather than the proliferation of cells.

The high frequency of the PI3K pathway alterations in cancer has directed the research in the development of PI3K inhibitors. Many of these targeted therapies are currently in clinical trials.[12] This pathway is unique in that every major node is frequently mutated or amplified in a wide variety of solid tumours. RAS and PIK3CA mutations in the colon are observed at a frequency of 7.3%. This suggests that constitutively active KRAS and PIK3CA probably function synergistically in the colorectal epithelium to offer an important selective advantage. A possible mechanism is that PIK3CA mutations arise first in these tumours and afterward RAS facilitates activation of the mutant PIK3CA.

### 1.5.3 A third downstream pathway acts through Ral, a distant cousin of Ras

RAL guanine nucleotide dissociation stimulator (RalGDS) is a downstream signalling protein of KRAS that functions as a GTP/GDP exchange factor to promote the GDP/ GTP conversion of RAS-like protein (RAL). This third of the three major effector pathways downstream of Ras involves a pair of Ras-like proteins termed Ral-A and Ral-B, which share 58% sequence identity with Ras and have the same functional activation by the replacement of GDP with GTP. The communication between Ras and Ral is mediated by Ral guanine nucleotide exchange factors (Ral-GEFs) and the resulting activation of RalA and RalB proteins allows them to regulate targets further downstream in the signalling circuitry. KRAS also regulates TIAM1 and RAC1- specific guanine nucleotide exchange factors, to activate RAC1 signalling pathways that affect cell shape, migration, adhesion, actin cytoskeleton formation, endocytosis, and membrane trafficking.

### 1.5.4 Posttranslational modifications that regulate Ras membrane association and subcellular localization

A post-translational modification controlling the localization, activity, and protein–protein interactions of small GTPases, including the Ras superfamily in all human cells is protein prenylation. Protein prenylation is a covalent post-translational modification present in all eukaryotic cells. This process comprises the attachment of either a farnesyl or a geranylgeranyl isoprenoid in the protein. It is essential for the proper cellular activity of numerous proteins, including protein-membrane interaction.

The covalent attachment of either a farnesyl (15 carbon) or a geranylgeranyl (20 carbon) isoprenoid group of the KRAS with the cell membrane is catalysed by four prenyltransferases,

namely farnesyltransferase (FTase), geranylgeranyltransferase type I (GGTase-I), Rab geranylgeranyltransferase type II (GGTase-II), and recently discovered geranylgeranyltransferase type III (GGTase-III).

KRAS is synthesised primarily as a cytosolic and inactive protein. It terminates in a C-terminal CAAX tetrapeptide motif comprised of an invariant cysteine residue to which the lipid is attached, followed by two typically aliphatic residues (AA), and the C-terminal residue (X) that contributes to prenyltransferase substrate specificity. This CAAX motif is necessary and sufficient to signal a series of posttranslational modifications that enhance the hydrophobicity of Ras which in sequence will facilitate the membrane association.

The first step in this process is catalysed by cytosolic farnesyltransferase (FTase)-mediated covalent addition of a C15 farnesyl isoprenoid to the cysteine of the CAAX motif, followed by Ras converting enzyme 1 (Rce1)-catalysed proteolytic removal of the AAX peptide, and finally isoprenylcysteine methyltransferase (ICMT)-catalysed carboxymethylation of the now terminal farnesylated cysteine. Recent studies have developed FTase inhibitors (FTIs) as anticancer agents, and currently, there are five of them (antroquinonol, tipifarnib, lonafarnib, BMS-214662, and L778123) that have experienced clinical trials on various kinds of tumours. They can prevent all three modifications.[11,12] However, KRAS becomes geranylgeranylated by GGTase I and active when cancer cells are treated with FTIs.

**2. Disruption of KRAS and membrane interaction as a potential target therapy**

Due to its peculiarities, KRAS has drawn attention for over 3 decades. Its crucial role in the development of cancer, its biological structure, and the variety of downstream signalling and effects have driven our concerns (Fig. 2.1). Efforts have been made in the direction of a single pathway inhibition or the blockage of interaction between KRAS and the cell membrane. As mentioned before, the first and most important step in the activation of this protein is the anchorage in the cell bilipid layer.

Efforts have been made in the inhibition of a basic function of KRAS: its affinity to the cell membrane. As mentioned before, a crucial step in the protein’s activation is the membrane localization to attach to the GEFs which are recruited by the ‘bridge’ Grb-Shc/SOS.

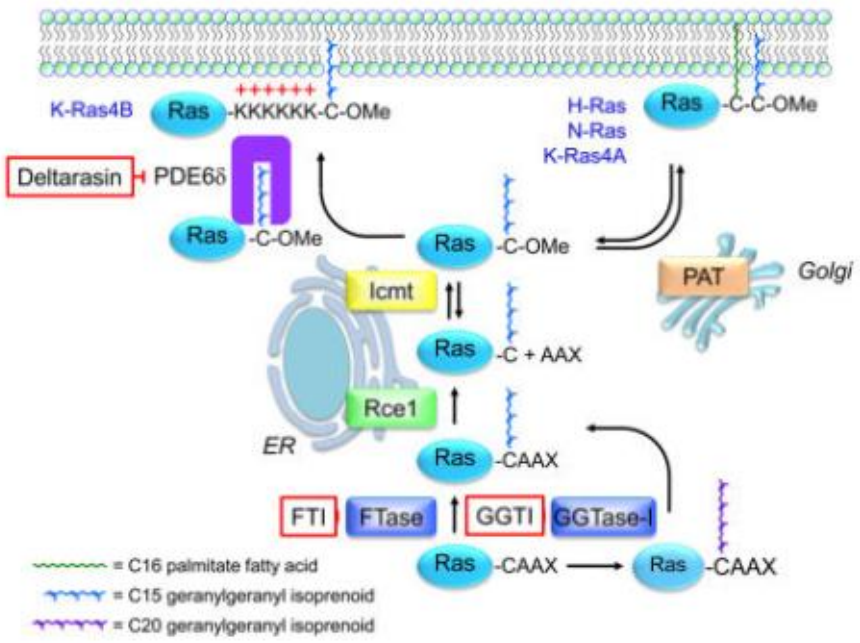


**Fig. 2.1**: The posttranslational modifications of RAS protein and possible druggable targets that inhibit this process[13]

In general, all Ras proteins are modified by FTases in cells. However, upon FTase inhibition by FTI treatment, KRAS4B and NRAS can be modified instead by the addition of a C20 geranylgeranyl isoprenoid. This reaction is catalysed by the related enzyme geranylgeranyltransferase I (GGTase I). The CAAX-signalled modifications alone are not sufficient to promote Ras trafficking and association with the inner face of the plasma membrane. Ras proteins possess a second membrane targeting element composed of either a polybasic amino acid stretch (K-Ras4B) or cysteine residues that undergo reversible acylation by the fatty acid palmitate.[14] This led to the idea that inhibition of FTase alone is not enough and the induction of dual inhibition of both FTase and GGTase I is required.[15]

### 2.1 FTase Inhibitors (FTIs)

Several peptidomimetic FTIs are developed with efficient competition towards peptide substrates, by blocking fully or partially the peptide site and the exit groove, where the displaced prenyl group of lipidated product binds. Potent FTIs such as tipifarnib and lonafarnib, which progressed to Phase III clinical trials, with the latter, under the name Zokinvy™, have been approved in the USA for Hutchinson-Gilford Progeria Syndrome (HGPS) and processing-deficient progeroid

laminopathies.[16] Furthermore recent studies have developed FTIs as anticancer agents and currently there are five of them (antroquinonol, tipifarnib, lonafarnib, BMS-214662, and L778123) have experienced clinical trials on various kinds of tumors.[14]

### 2.2 GGTase-I Inhibitors (GGTIs)

GGTase plays a critical role in the process of posttranslational modification of KRAS. Most GGTIs are protein-substrate-competitive inhibitors, with the structures derived from the tetrapeptide of the C-terminal sequence CAAX. These inhibitors are based on the C-terminal CAAL sequence of many geranylgeranylated proteins and they contain peptidomimetics that show exceptional selectivity for GGTase-I over the closely related enzyme protein farnesyltransferase (PFTase).[17,18]

Another idea was the inhibition of prenyltransferases. They commonly interact with the prenyltransferases via coordination of the zinc ion, the lipid-binding and/or CAAX substrate-binding site, the exit groove, or specific to a particular enzyme-binding site. Nonetheless, their intrinsic high affinity to other enzymes makes them less attractive as molecules with potential therapeutic properties.

GGTI-2418 is a peptidomimetic small molecule inhibitor of GGTase I which in a clinical trial phase 1 showed a relatively safe profile and tolerability at all tested dose levels with some evidence of disease stability.[19]

### 2.3 Selected Dual Inhibitors of FTase and GGTase-I

A problem that occurred after the blockage of FTase with FTIs was the activation of cellular proteins such as KRASB. This protein is activated through geranylgeranylation when the FTase activity is impaired, resulting in reduced treatment efficacy of FTase inhibitors. It appeared that targeting isoprenylation as a means of controlling cancer cell growth requires the inhibition of both GGTase and FTase for optimal activity.[15]

A novel study by Kazi et al. used a RAS C-terminal mimetic dual FTI and GGTI, called FGTI-2734, to overcome this geranylgeranylation-dependent resistance to FTIs.[20] It appeared that this molecule inhibited membrane localization of KRAS in pancreatic, lung, and colon human cancer cells and also induced apoptosis and inhibited the growth in mice with mutant KRAS-dependent human tumours. Importantly, FGTI-2734 inhibited the growth of xenografts derived from several pancreatic cancer patients hosting mutant KRAS (two G12D, two G12V) tumours.

Furthermore, Jiazhi et al. in 1999, to overcome the hurdle of geranylgeranylation of KRAS when FTase is blocked, combined non-thiol-containing peptidomimetics such as GGTI-2154 and FTI-

2148 with cytotoxic agents such as Cisplatin, Taxol, and Gemcitabine and demonstrated that this combination therapy is more beneficial than monotherapy in inhibition of human tumour growth.[16] This idea was based on the chemical properties of these peptidomimetics. Specifically, FTI-2148 is highly selective for FTase ($IC_{50}$, 1.4 nm) over GGTase I ($IC_{50}$, 1700 nm), whereas GGTI-2154 is highly selective for GGTase I (21 nm) over FTase ($IC_{50}$, 5600 nm).

In conclusion, targeting one of the basic properties of the KRAS protein, such as the prenylation, which is necessary for protein-membrane interaction, seems promising. This procedure can be blocked in multiple ways because there are at least three main reactions that take place and are conducted from two enzymes FTase and GGTase I. Early, ongoing clinical trials have promising results and will probably proceed further.

**3. Incidence of mutant KRAS in overall human cancers**

In the RAS family, KRAS is the most frequently mutated gene, followed by NRAS.[21] The KRAS oncogene has been studied extensively in human malignancies since its discovery. Overall, KRAS is altered in 15.95% of cancers, with pancreatic, lung, colon adenocarcinoma, colorectal, and rectal adenocarcinomas having the greatest prevalence of alterations. It is the most commonly mutated member of the RAS family and is considered to be the most common oncogenic gene driver in human cancers. Subsequent intensive sequencing of the cancer genome at The Catalog of Somatic Mutations in Cancer (COSMIC), which is the most comprehensive database on human tumour mutations currently available, has revealed that, despite the identification of over 500 validated cancer genes, the three *RAS* genes (*HRAS*, *NRAS,* and *KRAS*) still comprise the most frequently mutated oncogene family in human cancers (Fig. 3.1).[22]

KRAS mutations are most common in PDAC, CRC, and NSCLC. In particular, the prevalence of KRAS mutations is about 30% in non-small cell lung cancer (NSCLC), 30–50% in CRC, 80% in pancreatic adenocarcinoma, and 45–54% in extrahepatic cholangiocarcinoma. The profile of KRAS mutations differs significantly among different cancer types. Ivan Roa et al. found in a study of 106 CRC cases that KRAS was mutated in 46 (42.2%) of them.[23] The most dominant point mutations are at codons 12 (80.4%), G12D (39.1%), G12V (24.2%), G12S (6.5%), G12A (4.3%), G12C (4.3%), G12R (2.1%) and 19.6% at codon 13, the G13D. The results of this study demonstrated no differences in the frequency and distribution of mutations by gender, age, primary versus metastatic tumours, or tumour location.

| Tumour types | Sample | Total rate |
|---|---|---|
| Pancreatic adenocarcinoma | 1207 | 67.61 |
| Colorectal adenocarcinoma | 3953 | 35.77 |
| Nonsmall-cell lung cancer | 7135 | 20.42 |
| Cholangiocarcinoma | 1072 | 12.69 |
| Uterine endometrial carcinoma | 1907 | 14.11 |
| Testicular germ cell cancer | 506 | 11.66 |
| Cervical squamous cell carcinoma | 607 | 4.28 |
| Myelodysplastic | 6940 | 3.83 |

**Table 1:** Data sources from cBioPortal.org[24] showing the total rate of KRAS mutation in each type of cancer. The highest percentage is observed in PDC. CRC is the second most frequent cancer with mutations in the KRAS gene.

Searching the genome database of cBioPortal.org for the incidence of KRAS mutation in overall human cancers, the results are accordant to the ascendancy of the PDAC for mutant KRAS, with 67% of the cases containing the mutation and next is CRC where 35 % of the samples mutations were identified (Table 1).

Notably, the incidence of KRAS mutations ranges between 25 and 35% in smokers and 5% in nonsmokers and thus, smoking is usually considered to be a relevant factor. Nevertheless, the profiles of KRAS mutations are distinct in smokers and nonsmokers, and not all mutations in KRAS are driver mutations.

In addition to the different mutation subtypes in different cancer tissues, KRAS mutations also exist with different co-mutations. This usually influences the function of KRAS and the occurrence and development of tumours. The most frequent co-mutation with KRAS is the p53 gene (TP53) mutation which accounted for 39,4% of all co-mutations in NSLC.

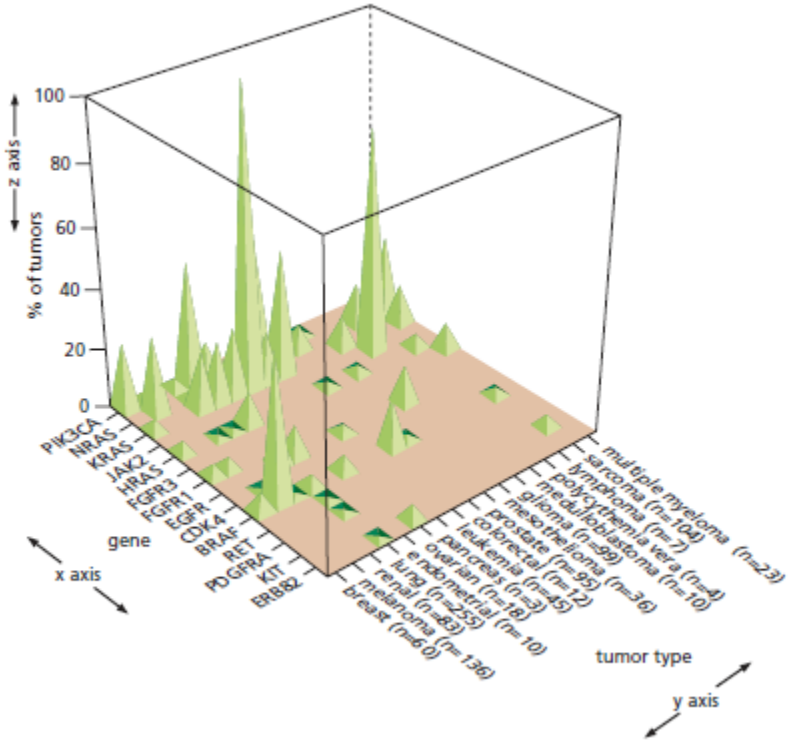


**Fig. 3.1:** Frequencies of oncogene activation in various human tumours. This three-dimensional histogram illustrates the variability with which various oncogenes are found in the mutant, activated forms in the genomes of human tumors[25]

### 3.1 Type and Frequency of KRAS mutations

KRAS mutations are dominated by single-base missense mutations. 98% of these are found at codon 12 (G12), codon 13 (G13), or codon 61 (Q61) (Fig. 3.2). They are predominantly clustered in exon 2 (codons 12 and 13), exon 3 (codon 61), or exon 4 (codon 146). These amino acid changes result in a permanent KRAS-GTP activation which consequently leads to tumour initiation and progression. In particular, more than 90% of KRAS mutations occur at glycine 12. This amino acid lies in the P-loop region of the protein and plays a pivotal role in stabilising nucleotide binding.

Given that the most common KRAS gene point mutation is glycine at position 12 (G12), followed by glycine at position 13 (G13), and glutamine at position 61 (Q61) the study is concentrated in the G12 amino acid. At this position occur 15 different point mutations, including G12A, G12D, G12F, G12K, G12N, G12S, G12V, G12Y, G12C, G12E, G12I, G12L, G12R, G12T, and G12W. Among these, G12D mutation accounts for about 41% of all the G12 mutations.[23]

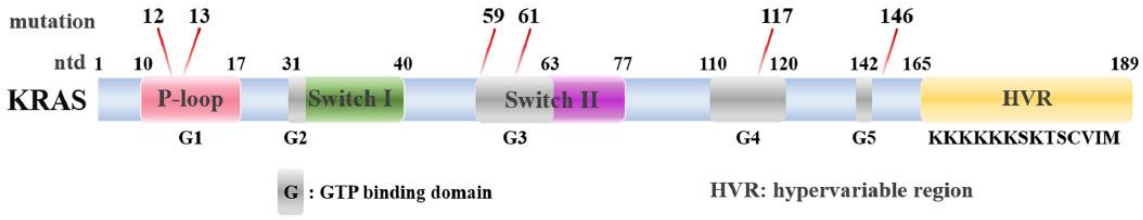


**Fig. 3.2:** KRAS structure domain and the residues 12, 13, 61,146 with the most frequent mutations in human cancers. In residues 69 and 117 have been observed mutations in some forms of cancers, but are extremely rare and without clinical significance[26]

In different clinical studies, cancers from many sites, independent of their stage, analyses were made for KRAS mutations and the type of mutation. The overall rate of mutant KRAS was approximately 42%, in almost 1 of 2 samples codon 12 was mutated and the most frequent nucleotide substitution was G12D, followed by G12V (Table 2).

### 3.2 KRAS Mutation-dependent signalling and biochemistry

As a result of the biochemical heterogeneity of the different mutations in the KRAS gene, not all mutant KRAS proteins affect the downstream signalling similarly and survival. Li et al. suggested that different mutations impact different oncogenic signalling at a unique level, and they promoted the ‘sweet spot’ theory, in which there is a narrow window of RAS mutations that can lead to oncogenesis. There are specific mutation patterns that can achieve the ideal level of signalling, essential for tumorigenesis.[5]

Codons 12 or 13 KRAS mutations result in base changes that lead to amino acid substitutions that lock the KRas protein in an active state.[26] The heterogeneous behaviour of mutant KRAS proteins implies that potential therapeutic interventions may need to take into account the contextually mutant KRAS expressed by the tumour. For example, Ihle NT et al., showed in a study of SCLC cell lines with mutant KRas G12D that the pathways of phosphatidylinositol 3-

kinase (PI-3-K) and mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) signalling were predominantly activated, whereas those with mutations of G12C or Gly12V had activated Ral signalling and decreased growth factor–dependent Akt activation.[27]

| | 13 | 14 | 15 | 38 |
|---|---|---|---|---|
| n = | 102 | 1267 | 108 | 1063 |
| Mutation rate% | 41(40.1%) | 505(40%) | 47.22% | 412(38.8%) |
| **Relative mutation distribution (%) by codon** | | | | |
| codon 12 | 75% | 344(27%) | 29.60% | 63.7% |
| codon 13 | 23% | 115(9.1%) | 8.30% | 22.8% |
| codon 61 | 2% | 19(1.5%) | 0.90% | 1.1% |
| codon 146 | | 40(3.2%) | 8.30% | 8.7% |
| other codons | | | 1.80% | |
| **Relative mutation distribution (%) by nucleotide substitution** | | | | |
| G12D | 48% | 157(12%) | | 123(29.9%) |
| G12V | 36% | 93(7.3%) | | 85(20.6%) |
| G12C | 6% | 43(3.4%) | | 27(6.6%) |
| G12S | 6% | 12(1.0%) | | 13(3.2%) |
| G12A | 4% | 20(1.6%) | | 10(2.4%) |
| G12R | | 7(0.6%) | | 4(1.0%) |
| G13D | 100% | 103(8.1%) | | 93(22.6%) |
| G13C | | 2(0.2%) | | 1(0.2%) |
| Q61H | | 7(0.6%) | | 1(0.2%) |
| Q61L | | 4(0.3%) | | 3(0.7%) |
| Q61R | | 2(0.2%) | | 1(0.2%) |
| Q61K | The only one case | 4(0.3%) | | |
| A146T | | 21(1.7%) | | 29(7%) |
| A146V | | 11(0.9%) | | 7(1.7%) |
| A146P | | 3(0.3%) | | |

**Table 2:** The mutation rate of KRAS in different clinical studies for overall cancers, the distribution of mutations by codons 12, 13,61, and 146, and the distribution by nucleotide substitution[26]

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As mentioned before, this gene contains 4 coding exons, and 1 non-coding exon, of which exon 2 has the highest mutation rate. Many studies have proved its direct association with the occurrence of poor prognosis and drug resistance. The most common KRAS point mutations are on residues 12,13 and 61. Oncogenic mutations in the three most commonly altered codons (12, 13 and 61) of RAS isoforms all impair GAP mediated GTP hydrolysis, rendering RAS preferentially GTP bound and active.[27] The nature of the substitution at each of these positions can also impact this cycling, not only by disrupting the GTPase activity, but also by deviating the downstream pathway. As an example, both valine and aspartic acid substitutions at G12 reduce the affinity for the effector RAF, yet a valine substitution at this position activates RAF better than an aspartic acid because it reduces GTPase activity more potently.

Moreover, the mutations in G12 and Q61 residues create a greater sensitivity to neurofibromin 1-mediated hydrolysis, whereas mutations in the G13 residue are partially sensitive to neurofibromin 1, a kind of GAP. In addition, cells containing KRAS G12C or G12V have increased levels of RAS-related protein (RAL) A/B signalling and decreased levels of phosphorylation of protein kinase B (AKT) compared with cells with other KRAS mutations or wild-type cells (Fig. 3.3). However, cell lines with KRAS G12D have higher levels of phosphorylated AKT and last, according to the binding affinity of KRAS with the effector RAF, the mutation can be divided into two classes: those with high affinity to RAF which are G12A, G12V, G12R, Q61H, and Q61L and those with low-affinity G12R, G12D, and G12V.

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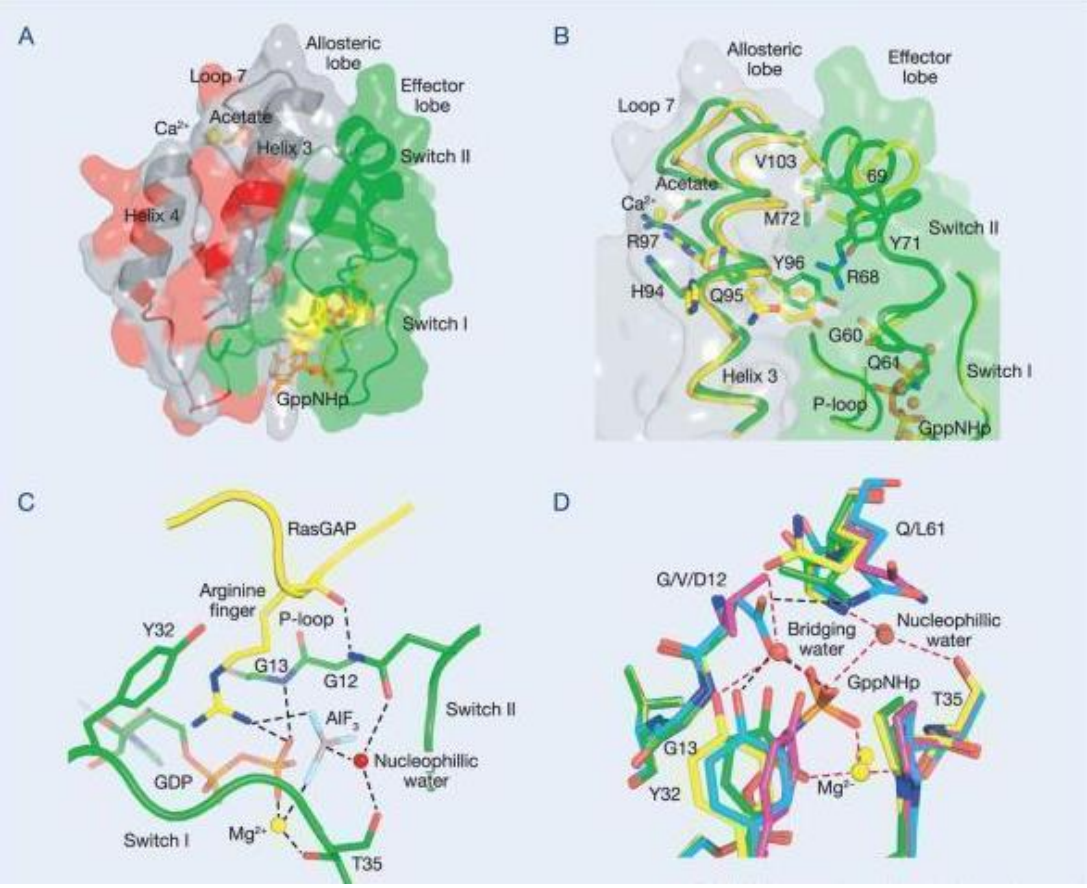



**Fig. 3.3**: A. The catalytic domain of Ras. B. The allosteric switch shows some of the key residues involved. C. The active site in the Ras/RasGAP complex. G12, G13, Y32 T35, and Q61 are shown in a stick. The Mg2+ ion is depicted as a yellow sphere and the nucleophilic water molecule as a red sphere. D. The active site for intrinsic hydrolysis in Ras. The wild type is shown in yellow, G12V in magenta, G12D in cyan, and Q61L in green. Note that in G12D the side chain of D12 replaces the bridging water molecule[28].

As a result of these biochemical peculiarities, there are different sensitivities in targeted therapies of mutant KRAS. For example, the mutation G12C has been proven sensitive to covalent inhibitors, when G12D is not. Furthermore, in terms of metastasis, the type of mutation seems to define the site of metastasis. A study by Jia Y et al. revealed that patients harbouring G12V mutations often have pleuropericardial metastases, while patients harbouring G12C and G12D mutations more preferentially have bone metastases.[29]

### 3.3 KRAS mutations in Colorectal Cancer (CRC)

Cancer is the gradual accumulation of mutations in genes leading to an increase in cell proliferation. The 'Vogelgram' (Fig. 3.4) was proposed in 1990 as a model of cancer development and it associated specific gene alterations to different stages of CRC development. It also identified the occurrence of multiple, sequential, functional, and structural genetic alterations that, in the aggregate, cause a temporal progression from early lesions to late CRC formation. More specifically, the core of the adenoma-carcinoma sequence is represented by point mutations in the KRAS gene that unleash a second round of clonal growth, which allows an expansion of cell numbers and, as a consequence, the evolution from a small to a large adenoma.[30]

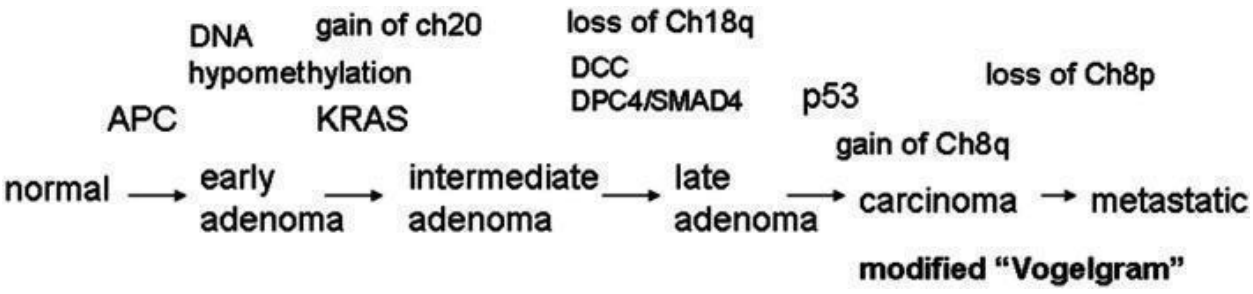



**Fig. 3.4:** The 'Vogelgram': The sequential order of cancer progression. In this diagram was emphasised the importance of mutation accumulation, rather than the sequential order. The KRAS mutation occurs early in the development of the carcinoma, specifically in the stage of the early adenoma[30].

KRAS oncogenic activation is a crucial event, resulting in the malignant transformation of the colonic epithelium. Roughly 3000 KRAS point mutations have been reported in CRC but the activating point mutation of the KRAS oncogene is at codon 12 (exon 2) and it is considered the initiating event in the majority of CRC cases (83%)[31]. The percentage of KRAS mutations in CRC is illustrated in Fig. 3.5.

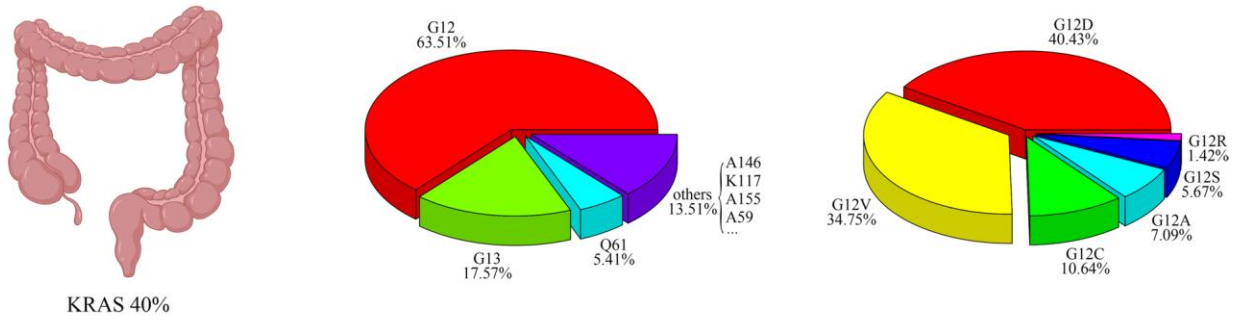



**Fig. 3.5:** Percentage of KRAS mutations in CRC and the diversity of KRAS alleles. In CRC KRAS is mutated in approximately 40% of the cases, independent of the stage, the most common residue is G12 and the most frequent mutation is the substitution of glycine by aspartic acid. Data acquired from The Cancer Genome Atlas (pan-Cancer) from cBioPortal[24]

The substitutions G12D and G12V are located near the GTP-binding site. This causes an interference with GTPase activity by impairing the GAP-mediated hydrolysis of GTP to GDP and thereby locking the KRAS protein in a hyperexcitable state. As a result, these mutations are driver mutations for cancer development.

Polymerase chain reaction (PCR)-based assays are usually employed to investigate the KRAS mutational status at the main hotspot mutation. On the contrary, next-generation sequencing (NGS) assays which allow full exon analysis, can be used to detect uncommon mutational profiles, but this technique is still of unknown clinical effectiveness.

KRAS mutations in CRC have a great impact in tumour development, growth, and resistance to chemotherapy. Identifying RAS mutation status is necessary for all stage IV CRC patients, based on recent guidelines. The reason for this is that KRAS mutant cancers develop resistance to anti-EGFR treatment.

Another interesting evidence of KRAS mutations is the population-based incidence. Studies have proved that KRAS mutations are population-based, indicating the environmental and hereditary role. In Caucasians, the frequency of CRC with KRAS mutations is equal to 38%; in Asians, it is close to 40% and, in Africans, it is only 21%.

### 3.4 Intratumoral heterogeneity of KRAS in CRC

Many studies have suggested a discrepancy in the results according to the intratumoral heterogeneity of KRAS in CRC specimens collected after surgical resection. In a study sample from 102 metastatic CRC patients were collected[33]. DNA from 2 or 3 areas from the primary tumour and 1 area of metastatic tissue was obtained from formalin-fixed paraffin-embedded specimens and testing for KRAS mutation in codons 12, 13, and 61 was performed by Pyrosequencing (Fig. 3.6). It was performed in primary and metastatic tumour samples. 100% concordance was observed regarding KRAS mutational status (wild-type vs. mutated) in the various areas of the primary tumour. This study proved that KRAS status is highly homogeneous throughout primary CRC tumour areas and consistent between the primary tumour and metastatic tissue in the same patient.

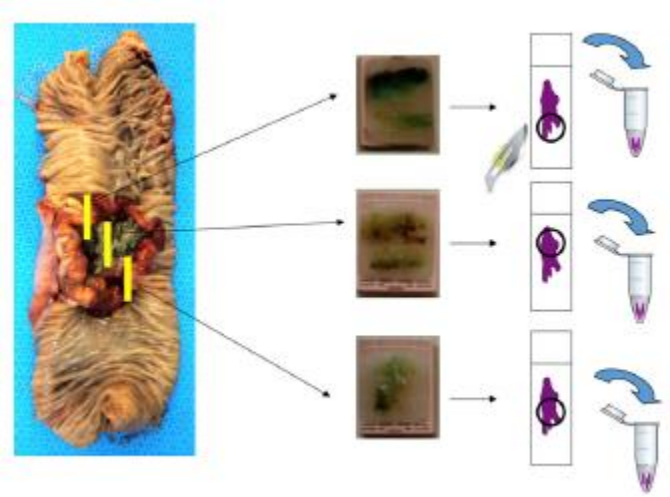


**Fig. 3.6:** Representative scheme of routine macroscopic sampling of primary tumours for assessing intratumoral heterogeneity between 2 or 3 areas of the primary tumour, depending on tissue availability. No intratumoral KRAS heterogeneity was detected in colorectal adenocarcinoma.[32]

Overall, in the setting of anti-EGFR treatment, the results of this study suggested that testing KRAS mutations in only 1 area of the primary or metastatic tissue are suitable for predicting the response to anti-EGFR treatment and guiding treatment decisions.

On the other hand, Kosmidou et al., examined 171 resected CRC adenocarcinomas with the method of PCR and subsequent pyrosequencing, and revealed a highly heterogeneity of 50,7%. Differences, such as double KRAS mutations in the same sample were detected between tumour centre and periphery of the specimen. More specifically, 24 out of 75 KRAS mutant samples were bearing a mutation in codons 12 and 13 simultaneously, 10 samples had a KRAS mutation in the tumour centre, but no KRAS mutation in the periphery region and 5 had a double mutation in the periphery, while a KRAS single mutation was carried in the centre.[33]

From the above no safe result can be extracted. More studies are required in order to establish the intratumoral behaviour of KRAS mutations.

### 3.5 KRAS and CRC sidedness

The mutational status of KRAS can be different in CRC regarding the tumour location. These differences have been investigated in studies comparing right (RCC) vs. left-sided cancer (LCC)[29]. RCC encompasses cancers arising in the cecum, ascending colon, and hepatic flexure. Accordingly, LCCs are cancers developed in the splenic flexure, descending colon, and sigmoid colon. Molecular profiling of CRC revealed different molecular characteristics in these two cancers (Fig. 3.7).

Salem et al. analysed the genomic profiles of 612 consecutive CRCs by performing Direct sequencing on genomic DNA isolated from FFPE tumour specimens and demonstrated KRAS mutations in 61%-71% of RCC and at a lower frequency in LCC 30%-36%[34].

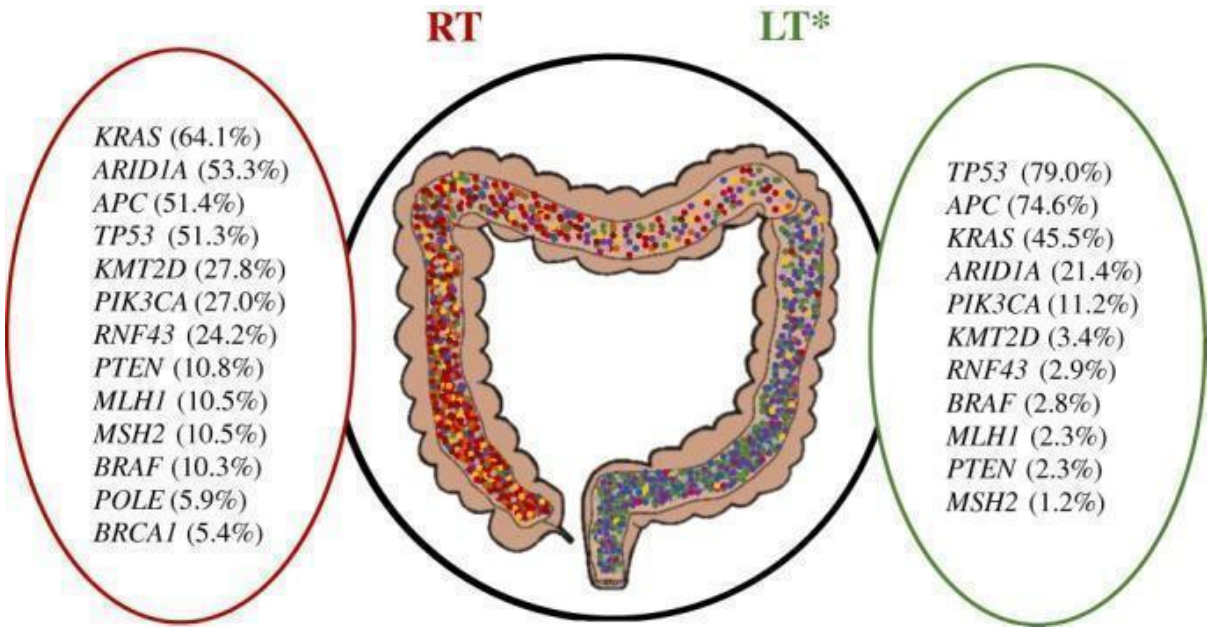



**Fig. 3.7:** Main significant gene mutation rates observed between RT (right side tumour) and LT (left side tumour) in colorectal cancer (CRC) using next-generation sequencing (NGS)[34]

These two cancers represent clinically and molecularly distinct entities with significant differences in their prognosis and treatment outcomes. The gene analysis of these two types of cancer revealed molecular aberrations with several genes including BRAF (10.3% vs. 2.8%), KRAS (64.1% vs. 45.5%), PIK3CA (27% vs. 11.2%), and RNF43 (24.2% vs. 2.9%) for RCC and LCC irrespectively.

Tumour-sidedness has emerged as a prognostic and predictive biomarker in metastatic CRC (mCRC), with evidence of poorer outcomes in right-sided mCRC and variable responses to biological therapy based on the site of origin of the tumour. In rectal cancers, data obtained by the analysis of KRAS exon 2 only revealed KRAS mutations in 42% of patients; data from exons 3 and 4 are still lacking.

### 3.6 Identification of KRAS mutations in CRC

Given the critical role of KRAS in the prognostic evaluation and therapeutic decision-making, KRAS mutations should routinely be tested in the diagnosis of CRC. Currently, direct sequencing, with polymerase chain reaction (PCR), followed by dideoxy sequencing, is the gold standard

method for detecting mutations. This method has a high sensitivity but requires a high allele frequency of mutation (10–30%).[34]

Another method is the TheraScreen KRAS kit (Qiagen), a test based on amplification refractory mutation system (ARMS) technology. This kit detects seven mutations in codons 12 and 13 in the KRAS gene and has high sensitivity and specificity. Furthermore, there is available StripAssay (Vienna Labs) a mutant-enriched PCR followed by reverse hybridization, SNaPshot, and TaqMelt PCR assay Cobas (Roche) but the cost is higher, albeit a lower detection threshold.

The inability to detect uncommon, mutated alleles is a limitation of these kits. Next-generation sequencing (NGS) can identify energy mutations in the KRAS gene. It is based on the concept of pyrosequencing but uses fluorescence markers to determine the sequence of DNA nucleotides. It is a revolutionary method that has the advantage of tracing unique or unusual mutations, by analysing the whole genome of the tumour cells. Several techniques are combined with the NGS, such as endoscopic ultrasound sonography. Samples from the liver or pancreas are directly analysed with the NGS to diagnose possible malignant lesions. An advantage is that it can be applied in very small samples, even some millimetres. On the other hand, the high cost of this technique is currently a limitation, but the accuracy of the results makes the NGS an attractive method for the detection of unique mutations.

Another emerging analytical technique is the Liquid biopsy which has the advantages of minimal invasiveness and rapid detection. The clinical application of liquid biopsy has been developed in each clinical stage of CRC. This technique is based on the detection of circulating tumour DNA (ctDNA) which is cell-free DNA released into circulation by tumour cells and can be isolated from plasma. It is a PCR-based method, such as allele-specific quantitative PCR. Nevertheless, circulating KRAS mutation detection with this method does not perfectly reflect the mutation burden of the primary tumour from which it originated. Generally, liquid biopsy is a promising field in CRC. It requires larger cohort studies to confirm its efficacy but for sure it can provide complementary information for cancer.

**4. KRAS Targeted therapy**

The current research in the KRAS pathways, its biochemistry peculiarities, and its crucial role in carcinogenesis have drawn the attention of researchers to create inhibitors of this oncogene. The development of valuable drugs to prevent RAS-driven oncogenesis was challenging for more than three decades and RAS was deemed ‘undruggable’. The therapeutic strategies under investigation to target KRAS mutations in CRC include therapy directed towards mutant KRAS,

targeting KRAS-membrane association, and the combined inhibition of downstream pathways (Fig. 4.1). In the era of personalised-individualised medicine this approach is promising.

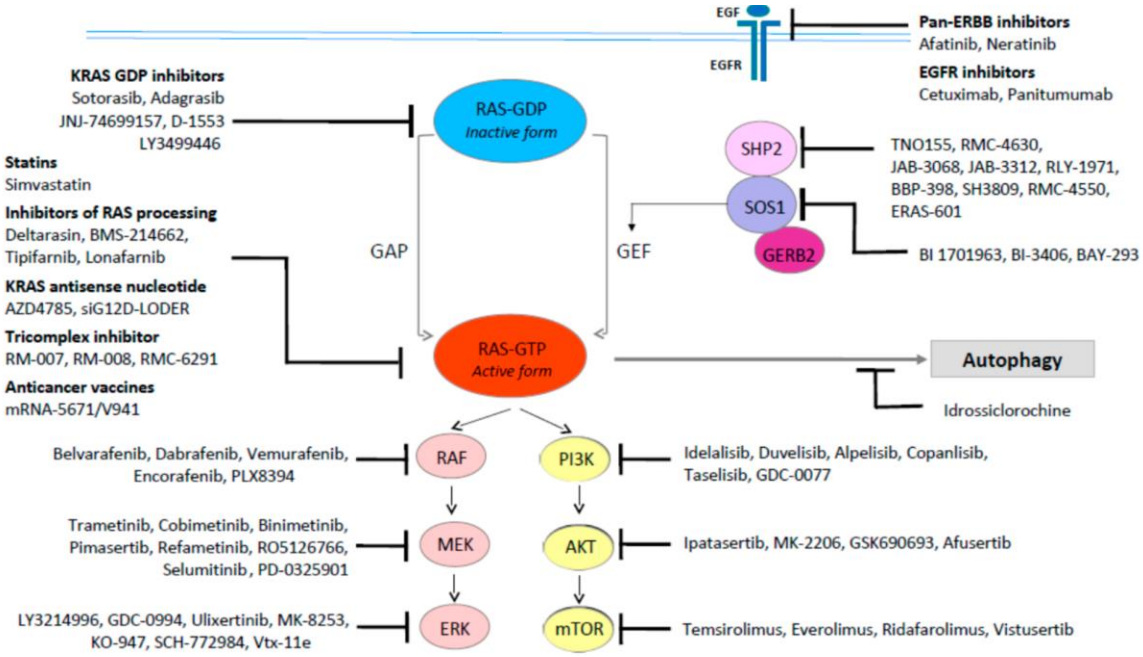



**Fig. 4.1:** KRAS targeted therapies that are currently in clinical trials[36]

### 4.1 KRAS direct inhibition

The cysteine residue that substitutes the glycine in the G12 position is an attractive target for covalent inhibitors. AMG 510 (Sotorasib) is the first FDA-approved specific, irreversible inhibitor of KRAS G12C. It traps the KRAS in the inactive GDP-bound state. It can selectively target KRAS G12C while preserving wild-type KRAS to circumvent the toxicity caused by the inhibition of all KRAS isoforms. Wild-type KRAS does not include cysteine in the active site, thus it is not inhibited by this covalent approach. AMG 510 has been shown in preclinical studies to inhibit phosphorylation of extracellular signal-regulated kinase (ERK), a critical downstream effector of KRAS, producing a durable complete tumour regression in mice. Although KRAS G12C is noted only in 1–3% of CRC, the recent promising clinical data of phase 1 trial CodeBreak100 (NCT03600883) in NSCLC breaks the assumption of KRAS being undruggable and brings promising results in the application in CRC patients.

Another potently covalent direct inhibitor of KRAS is MRTX849 (adagrasib). It works by irreversibly and selectively binding to KRAS in its inactive state, thus preventing cell proliferation and cancer growth and leading to cell death. The KRYSTAL-1 is a phase I/II clinical trial that showed remarkable anti-tumor efficacy of this agent against NSCLC, CRC, and other solid tumours. More precisely, adagrasib was investigated in combination with upstream inhibitors, like EGFR and SHP2, and downstream inhibitors, like mTOR and cyclin-dependent kinase 4/6 (CDK4/6). The result was a significant improvement in the anti-tumor activity of MRTX849 with a greater response rate (RR). Nonetheless, these results were better observed in NSLC than in CRC where the RR was lower. For this reason, ongoing trials in CRC are testing the combination of adagrasib with cetuximab (an anti-EGFR antibody) and TNO155 (an SHP2 inhibitor). These studies are underway.

MRTX1133 (Fig. 4.2) is another KRAS-directed investigational drug with more eclecticism against the KRAS G12D mutant form.

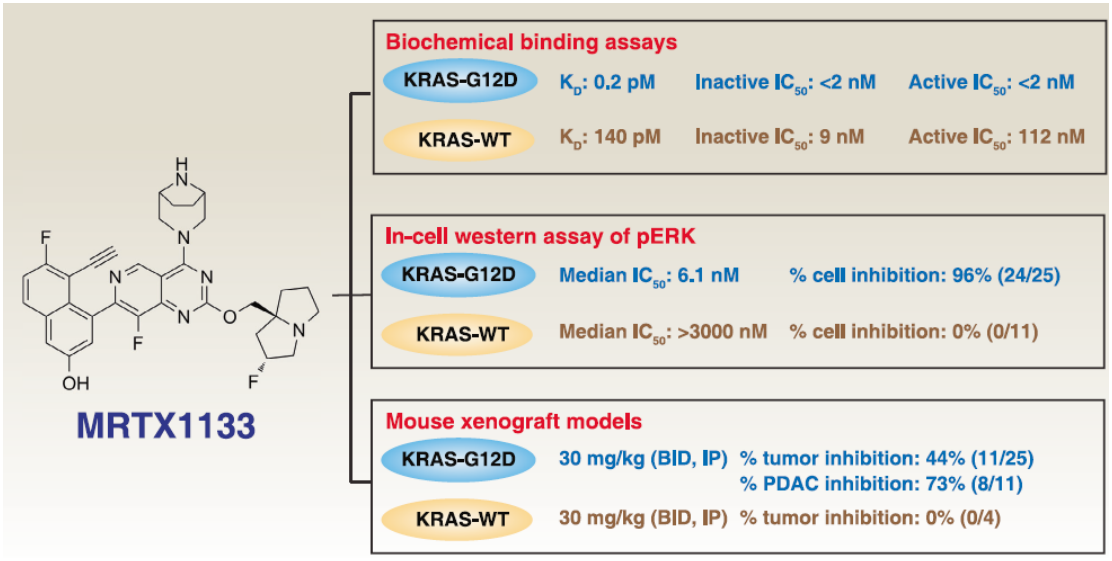



**Fig. 4.2:** MRTX113 is a selective inhibitor of KRAS-G12D protein. The activity and function of MRTX113 in targeting KRAS-G12D and KRAS-WT proteins were compared in cell-free systems, cellular, and xenograft models[36].

Xiaolun Wang et al. discovered MRTX1133 through a structure-based drug design strategy. It is a non-covalent inhibitor that binds to the inactive and activated states of KRAS-G12D protein,

resulting in KRAS pathway inhibition.[37] The preclinical studies demonstrated that it binds both the active and inactive forms and significant dose-dependent tumour regression was noted in animal models. There are currently phase I/II clinical trials that focus on NSCLC.[35]

MRTX1133 is highly selective for KRAS-G12D protein, more than 1000-fold higher than wild-type KRAS protein, resulting in an estimated KD of 0.2 pM for KRAS-G12D.

Several studies have investigated the role of pan-Ras inhibitors, which target the Ras-binding pocket, such as the in-silico discovery of the Kobe0065 compound[38]. This research took place at Kobe University and had some promising results. It binds to RAS-GTP and competitively blocks RAF binding, which furthermore inhibits the growth of cancer cells carrying activated RAS oncogenes in tumour xenografts. The problem that occurred was the high toxicity of these compounds, with several adverse effects in the preclinical models, because of the low specificity in KRAS protein.

### 4.2 KRAS indirect inhibition

When analysing the activation of KRAS after the membrane localization, a key reaction is the Shc-Grb2-SOS bridge between the protein and the cell membrane. This ‘bridge’ is thought to be a catalytic point before SOS exchanges GDP to GTP, thus activating KRAS. Studies have demonstrated that KRAS is free in the cytosol and many proteins gather forming clusters. The activation occurs in clusters of KRAS proteins, so the result of a single stimulus is amplified in many cascades.

#### 4.2.1 SOS1 inhibitors

As mentioned before, the activation of KRAS is mediated by guanine exchange factors (GEFs) which phosphorylate GDP to GTP, and this turns KRAS protein into an active state by binding in the pocket site. Several molecules have been developed to directly inhibit the SOS protein. A lipophilic pocket of SOS1 adjacent to the RAS-binding site is targeted and this could be occupied by small molecules, which could activate or block the SOS-RAS interaction. The BAY-293 and BI-3406, a more potent and selective SOS1 inhibitor, have been tested and demonstrated synergistic antiproliferative activity in a KRAS-mutant cell line, when combined with a KRAS G12C covalent inhibitor, such as sotorasib or adagrasib.

### 4.2.2 SHP2 inhibitors

SHP2 is a protein tyrosine phosphatase encoded by the gene PTPN11. Its tyrosine phosphorylation contributes to interaction with growth factor receptor-bound protein 2 (GRB2). It acts as a scaffold in the stabilisation of the 'bridge' between KRAS and the cell membrane, thus it has been speculated that by impeding the building of this bridge, KRAS-membrane interaction will be blocked. A study by Chen X et al. demonstrated that SHP2 inhibitors limited the proliferation of KRAS-mutant CRC in vitro and in vivo.[39] In some studies the MEK inhibitor trametinib was used in combination with the SHP2 inhibitor TNO155 to improve the efficacy in KRAS-mutant CRC. Other ongoing trials are the RMC-4630 which is in phase II and administers a molecule as a single agent (NCT03634982) and the clinical trial LY3214996 where an SHP2 inhibitor is used in combination with an ERK inhibitor for the treatment of patients with metastatic KRAS-mutant CRC.

## 4.3 Targeting downstream signalling pathways

The RAS/RAF/MEK/ERK and PI3K/AKT/mTOR are two of the most frequently dysregulated pathways in human cancer biology. These two pathways interact closely. They share common inputs and provide compensatory signalling when either one is inhibited, meaning that there is a crosstalk reaction between them. They also have feedback mechanisms This is the basic characteristic that demands a combination of therapeutic agents that block simultaneously both the pathways or the crossroads that connect them and inhibit the downstream signalling of a permanently activated KRAS. Multiple clinical trials are underway targeting several aspects of the RAS downstream pathway. In Table 3 are demonstrated trials focused in metastatic CRC (mCRC) and in Table 4 trials against specific KRAS mutations.

### 4.3.1 RAF-MEK-ERK inhibitors

KRAS activation causes dimerization and phosphorylation of its downstream RAF proteins, which induces RAF kinase activity and then contributes to the phosphorylation of MEK1/2. So the cascade begins with the activation of RAF protein, thus it is the first target of this pathway. LY3009120 is an RAF dimer inhibitor that effectively inhibits the activities of the BRAF/CRAF heterodimer and BRAF or CRAF homodimer by occupying both promoters in the dimer. In a preclinical setting, Vakana et al. proved that this pan-RAF inhibitor displayed an antiproliferative effect in KRAS-mutant CRC cell lines and inhibited tumour growth in KRAS-mutant xenograft models[40]**.** In the same context of RAF inhibition in CRC, another molecule was tested, lifirafenib (BGB-283), but it showed no responses to patients with KRAS-mutant CRC in the phase I clinical trial (NCT02610361).

The next step of the pathway is the activation of MEK, but due to the feedback-mediated RAF activation, the results were disappointing in KRAS-mutant CRC patients treated with MEK inhibitors. These molecules caused an increase in phosphorylated MEK. Although monotherapy with either MEK or RAF inhibitor is less promising in KRAS-mutant CRC, the combination of these two inhibitors established synergy in preclinical models of KRAS-mutant CRC cell lines.

| | | | |
|---|---|---|---|
| AMG510 | Direct KRAS G12C inhibitor | NCT03600883 | Phase 1 |
| JNJ-74699157 | Direct KRAS G12C inhibitor | NCT04006301 | Phase 1 |
| MRTX849 | Direct KRAS G12C inhibitor | NCT03785249 | Phase 1 |
| TNO155 | Direct KRAS G12C inhibitor | NCT03114319 | Phase 1 |
| Apoptotic, cell cycle and DNA damage pathways | | | |
| ABBV-621 + FOLFIRI + bevcizumab | TRAIL receptor agonist + Chemotherapy + anti-VEGF therapy | NCT03082209 | Phase 1/2 |
| Onvansertib + FOLFIRI + bevcizumab | PLK1 inhibitor + Chemotherapy + anti-VEGF therapy | NCT03829410 | Phase 1/2 |
| AZD1775 + Irinotecan | Wee 1 inhibitor + Chemotherapy | NCT02906059 | Phase 1 |
| Palbociclib + Binimetinib | CDK4/6 inhibitor + MEK inhibitor | NCT03981614 | Phase 2 |
| HDM201 + Trametinib | MDM2 inhibitor + MEK inhibitor | NCT03714958[a] | Phase 1 |
| Metabolic pathway | | | |
| TVB2640 | FASN enzyme inhibitor | NCT02980029 | Phase 1 |
| Vitamin C + FOLFOX ± bevacizumab | GAPDH enzyme inhibitor + Chemotherapy ± anti-VEGF therapy | NCT02969681 | Phase 3 |
| Immunotherapy combinations | | | |
| Binimetinib + Nivolumab ± Ipilimumab | MEK inhibitor + anti-PD1 therapy ± anti-CTLA4 therapy | NCT03271047 | Phase 1/2 |
| Utolimumab + Cetuximab + Irinotecan | 4-1BB/CD137 agonist + anti-EGFR therapy + Chemotherapy | NCT03290937 | Phase 1/2 |
| Hu5F9-G4 + Cetuximab | anti-CD47 therapy + anti-EGFR therapy | NCT02953782 | Phase 1/2 |

[a]TP53 wild-type tumor is also mandatory

**Table 3**: Selected ongoing clinical trials in RAS mutated metastatic CRC[41]

For example, NCT03284502, a phase I clinical trial is currently investigating the combination of belvarafenib, an RAF inhibitor, and cobimetinib, a MEK inhibitor in patients with locally advanced CRC.

The catalytic point of the cascade is ERK, thus it is speculated that inhibiting this protein may have greater efficacy in anti-tumor activity. In a phase I trial with 5 patients with advanced CRC (NCT01875705), the molecule GDC-0994, an ERK inhibitor, was evaluated as a single agent. Unfortunately, only one patient achieved stable disease and the remainder showed progression of the disease.

Other important strategies with promising results are the PLK-1 inhibitors and the KR12. Polo-like kinase 1 (PLK1) is a serine/threonine kinase that plays a key role in cell cycle progression via mitosis and DNA damage repair. This protein is overexpressed in KRAS-mutant cells. A selective PLK-1 inhibitor, called Onvansertib, is under clinical investigation as a second-line therapy in

metastatic CRC harbouring KRAS mutation along with a combination of FOLFIRI and bevacizumab. In phase II clinical trial NCT03829410 42% of patients achieved a partial response (PR) and a durable response in 67% of patients ranging from 6.1 months to 13.7 months.

| Compounds | Company | Mechanism | Clinical trial |
|---|---|---|---|
| AMG 510 | Amgen/Carmot Therapeutics | $KRAS^{G12C}$ inhibitor | NCT03600883 |
| MRTX849 | Mirati (ex Array) | $KRAS^{G12C}$ inhibitor | NCT03785249 |
| KRAS TCR | Gilead (ex Kite/NCI) | Anti-$KRAS^{G12D}$ engineered T-cell receptor | NCT03745326 |
| AZD4785 | AstraZeneca/Ionis | KRAS antisense oligonucleotide | NCT03101839 |

**Table 4**: Ongoing clinical trials involving direct targeting of KRAS[42]

KR12 is a pyrrole-imidazole polyamide indole-seco-CBI conjugate that recognizes and alkylates the adenine residues on the template strand at codon 12 (GTT and GAT), exon 2 of mutated KRAS. It is efficient in the mutations G12D and G12V in CRC cells harbouring these specific mutations. The growth suppression in G12D/G12V mutated CRC cells, ultimately induced senescence, and apoptosis. Significant tumour growth suppression was observed in xenograft models, with low toxicity in KRAS wild-type cells.

### 4.3.2 PI3K-AKT-mTOR inhibitors

Another peculiarity of the KRAS cascade is the crosstalk between the two downstream pathways PI3K and MAPK. The inhibition of one pathway can direct the compensatory stimulation of the other; hence, the inhibition of MAPK and PI3K is a promising strategy, but this combination in clinical trials had limited tolerability. Nonetheless, in KRAS-mutant CRC cell lines, specific PI3K inhibitors show the ability to overcome the resistance to MEK inhibitors and inhibit cell proliferation.

Because of the dose-dependent toxicity of these combinations, further studies are required to establish the efficacy of this strategy. Promising preclinical results have been observed in the combination of a PI3K inhibitor with a direct KRAS inhibitor.

### 4.4 Direct KRAS G12D inhibitor

Kotaro Sakamoto et al. have developed a K-Ras(G12D) inhibitory bicyclic peptide, called KS-58[43]. They presented evidence for anticancer activity against mouse xenografts derived from human pancreatic cancer cell lines PANC-1 stably expressing this gene with this specific mutation. Furthermore, they proved anticancer activity against mouse tumours derived from the colorectal cancer cell line CT26 stably expressing K-Ras(G12D). This newly discovered molecule has drawn the attention of researchers and seems to be the main research in many ongoing studies.

## 5. KRAS prognostic and predictive value

In many studies has been observed the impact of the KRAS G12D mutation on the prediction of targeted therapy. Moreover, KRAS mutation is a predictive marker of response to EGFR-targeting antibodies in CRC treatment[44]. EGFR-targeting antibodies are the first molecular-targeted therapy for CRC. They seem to have a response in only 10% of patients. Currently, the best first-line treatment for metastatic CRC in patients with RAS wild-type, is chemotherapy with an anti-EGFR molecule. Many randomised and single-arm trials of cetuximab or panitumumab have demonstrated that the EGFR monoclonal antibodies are only effective against tumours with wild-type KRAS. More precisely, KRAS exon 2 mutations are suggested to be the predictors of the lack of benefit to EGFR-targeted therapy**.**

In a meta-analysis combining the data from the 13 studies, it was found that KRAS mutations comprise a negative predictive marker for response to cetuximab with a specificity 95% but with low sensitivity 47%, indicating that cetuximab should be administered only to patients with wild type (KRASw) CRC.[45]

Furthermore, KRAS mutation status influences the choice of surgical techniques. More specifically, KRAS wild-type patients seem to have a better OS with a resection margin ≥ 1 mm. Studies have demonstrated that KRAS mutation is an independent predictor for positive resection margin. Besides, KRAS mutational status affects the outcomes of ablation in colorectal liver metastasis (CRLM). Patients with mutant KRAS have worse PFS after percutaneous ablation for CRLM, with 35% at 3 years when compared to 71% at 3 years for the KRAS wild-type patients ($P < 0.001$). These findings suggest that the KRAS mutation status should be considered when deciding the surgical therapies, especially for the CRLM patients[26].

In a cohort of 546 patients with stage III CRC, who had completed 6 or more cycles of adjuvant FOLFOX chemotherapy after curative surgery, Hye Eun Park et al. observed that G12D and G12V mutations were associated with poor RFS in both cohorts, while there was no difference in survival between the other G12 mutations and wild-type CRCs (Fig. 5.1). G12D/V mutations were also associated with a low tumour-infiltrating lymphocyte (TIL) density.

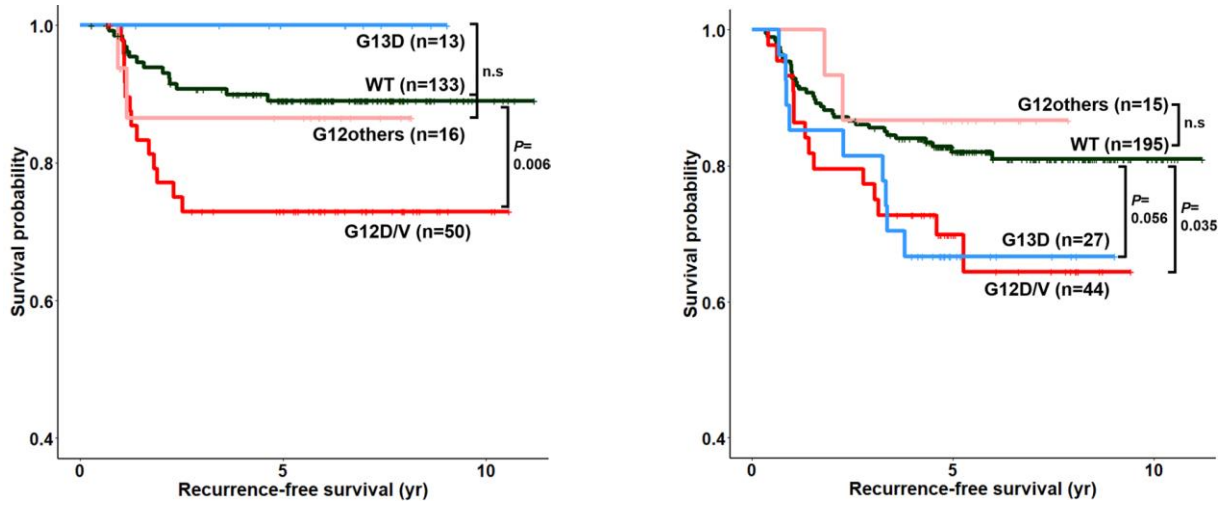



**Fig. 5.1:** Kaplan–Meier survival curve of the discovery cohort (left) and the validation cohort (right) based on recurrence-free survival (RFS) according to the KRAS mutation status[46]

In another study of 578 RAS-mutated CRC patients at stage I-IV the results according to the progression-free survival (PFS) and the overall survival (OS) showed that the KRAS G12D mutation had no statistically significant difference in PFS between other KRAS mutations but had better OS (Fig. 5.2)[47].

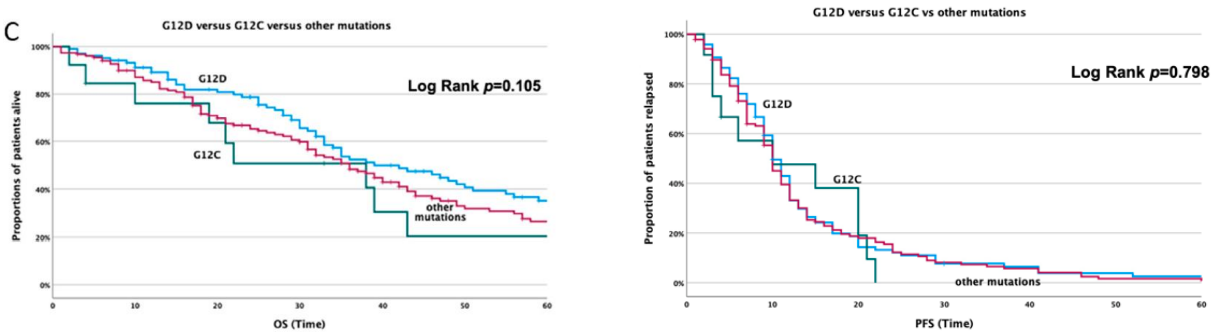



**Fig. 5.2:** OS (left) and PFS (right) regarding G12D, G12C and other KRAS mutations[47]

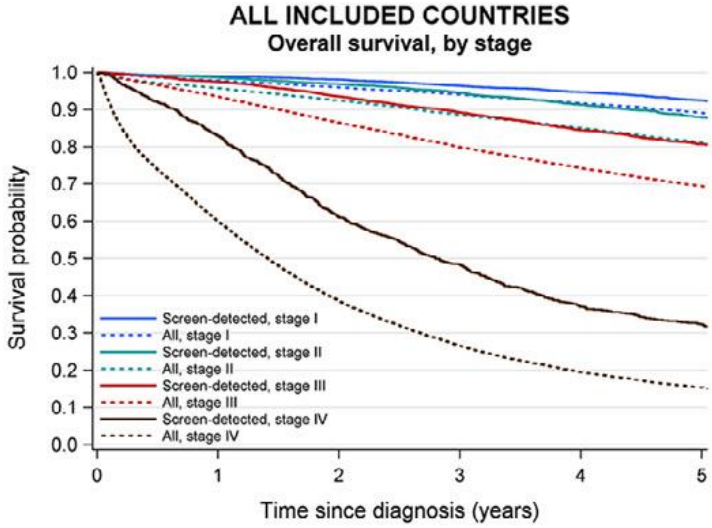
## Discussion

Cancer is one of the leading causes of morbidity and mortality in the world. Irreversible changes in the cellular genome are considered the definitive factor for carcinogenesis. More precisely, colorectal cancer is the third most common cancer worldwide. It has a compounding impact on health economics. Accordingly, to a population-based study with a cost-of-illness approach the economic burden of colorectal cancer in 2015 was €19 billion across Europe and an increase in expenditure on pharmaceuticals by 213% from 2009 to 2015 was observed.[48]

Moreover, in consideration of gender, CRC seems to have a higher preference in women compared to men, making it the second most frequent cancer type in females. In 2020 alone, more than 1.9 million (10.7 % of all cancers) new cases of colorectal cancer were registered in the World Cancer Research Fund International registry. Some countries in Europe are more vulnerable to CRC. For example, there is a considerable difference in the incidence of CRC between Hungary (45% ASR/100.000, ASR= age-standardised rate) and Croatia (35% ASR/100.000), making more profound the theory of inheritable genes that predispose towards cancer development. This theory can also be supported by the fact that these two countries have similar Human Development Indexes (HDI) (Hungary 0,845, Croatia 0,858) and it is noted that the HDI is an independent factor altering the incidence of CRC. It is estimated that almost 295 new cases of cancer per 100,000 people occur in areas with high HDI, compared with 115 new cases per 100,000 people in areas with low HDI (data collected from the 2020 WCRF registry). In this result, attention should be given to the fact that countries with low HDI usually lack the

advantage of a stable screening program, the availability of screening tools, and the prompt accessibility of people to healthcare systems that can provide adequate diagnostic accuracy.

Irrespective of the stage at diagnosis, patients with CRC have a 3-ys OS of 71.1% and a 5-ys OS of 63%. According to the stage of diagnosis, 5-ys OS for patients with stage I CRC is 89,1% and decreases at 81,2% for stage II and approximately 66,2% for stage III. In the case of metastatic (stage IV) cancer, (mCRC) 5-ys OS is only 15.4% (Fig. 5.3)[49].




No. of patients at risk

| | | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| Stage I | Screen-detected | 8380 | 8148 | 7736 | 5449 | 3775 | 2499 |
| | All | 18911 | 18226 | 17235 | 13485 | 9966 | 6963 |
| Stage II | Screen-detected | 4392 | 4252 | 3983 | 3036 | 2172 | 1416 |
| | All | 18522 | 17501 | 16331 | 13528 | 10306 | 7176 |
| Stage III | Screen-detected | 5221 | 5015 | 4601 | 3318 | 2293 | 1507 |
| | All | 21681 | 20046 | 17964 | 14140 | 10252 | 6973 |
| Stage IV | Screen-detected | 1476 | 1204 | 840 | 516 | 293 | 178 |
| | All | 16898 | 10038 | 6252 | 3753 | 2218 | 1306 |

**Fig. 5.3:** Overall survival of screen-detected colorectal cancer patients, and all colorectal cancer patients, by disease stage.

Additionally, KRAS has an impact on survival in mCRC. A study of 109 stage IV CRC patients established a significant difference between the survival outcomes of wild-type KRAS and mutant KRAS mCRC patients. In a period of 4 years, the study recorded 73% deceased mutant KRAS mCRC patients, while in the wild-type KRAS mCRC group death was recorded in 46% of patients.[50]

A more expanded study from Yu Imamura et al. compared the clinical outcome of 1261 colon and rectal cancers, based on their molecular characteristics[51]. The study focused on BRAF wild-type CRCs which accounted for 1075 samples. Pyrosequencing technology was applied targeting KRAS mutations in codons 12 and 13, which is a reliable method, as mentioned before. Overall 332 specimens harboured mutations in codon 12, 108 in codon 13 and 635 were KRAS and BRAF wild-type. Interestingly, mutation G12D was the most frequent in codon 12 (N=161). The results were according to higher colorectal cancer–specific mortality in patients with mutations in codon 12 than in those with mutations in codon 13. More precisely, patients with G12V mutation experienced the highest cancer-associated mortality and had the worst clinical outcome.

As for the treatment of CRC, the options remain stable. Either the cytotoxic doublet FOLFOX (5-fluorouracil and oxaliplatin) as adjuvant chemotherapy or the cytotoxic triplet FOLFIRI (5-fluorouracil (5FU) with irinotecan) or CAPOX (capecitabine plus oxaliplatin) benefit stage III and stage IV patients. Approximately, 4-5% of patients at stage II will benefit from chemotherapy. According to the IDEA study FOLFOX raises the 5-year OS at 82,4%, either with 3 or 6 months therapy and no statistically significant difference was observed with CAPOX[52]. EGFR antibodies Cetuximab, Panitumumab, and VEGF antibody Bevacizumab when used should be administered in combination with FOLFOX or FOLFOXIRI in selected, fit, and motivated patients where cytoreduction (tumour shrinkage) is the goal. Cetuximab alone, when administered in EGFR-expressing mCRC refractory to irinotecan, oxaliplatin, and fluoropyrimidines improved median OS at 6.1 compared with 4.6 months in patients treated with best supportive care[53]. With Panitumumab PFS was significantly improved at 13.8 weeks, compared with 8.5 weeks for BSC, although an OS benefit was not observed. In eleven RCTs with a total of 3178 patients with advanced colorectal cancer receiving Bevacizumab plus FOLFOX, the objective response rate and cancer control rate were statistically significantly higher than that of the FOLFOX group[54].

Future perspectives on the employment of these agents focus on the impact of KRAS mutation on their response. As mentioned before, KRAS mutation has proved a negative predictive factor for the administration of EGFR antibodies, by activating downstream pathways independently of EGFR and inducing primary resistance, thus attenuating the treatment options and complicating the management of patients with mutant KRAS CRC. In conclusion, mCRC patients with mutant KRAS are ineligible for anti-EGFR therapy.[55]

Nonetheless, the OS rates have not been elevated satisfactorily and the mCRC remains a global health issue, encountering the second most death numbers in comparison with other types of cancer. Fewer than 20% of mCRC patients will survive beyond 5 years from diagnosis. In the era of precision medicine, the KRAS component seems an attractive goal of treatment as it counts

for more than 40% of all CRC and its incidence increases accordingly to the stage of cancer. Based on the functional role of this protein, it could represent the ‘Achilles heel’ of the cancer cells. It is, therefore, possible that we are in front of a deciding discovery and, thus, research should focus on the therapeutic management of actionable mutations such as those involving the KRAS protein.

Currently, 6 ongoing clinical trials target the KRAS G12D mutation in a variety of human cancers, including CRC (Table 5). They are currently recruiting patients with a variety of solid malignancies and their results are eagerly awaited. Two phase III clinical trials in NSCLC have reported statistically significant benefits of two selective KRAS G12C inhibitors, sotorasib and adagrasib, leading to the approval of two drugs for NSCLC patients with the specific mutation. The goal is to collect information about the effectiveness of new molecules, their adverse effects, their impact on OS and PFS, and their toxicity or interaction with other compounds.

Secondary endpoints are clinically significant changes in ECOG, vital signs, and physical examination. Efficacy endpoints are Overall Response Rate (ORR), Duration of Response (DoR), Disease Control Rate (DCR), Progression Free Survival (DoR), and OS. These studies will also examine the Cmax (Maximal plasma concentration) of the drugs.

| | Title | Status | Study Results | Conditions | Interventions |
|---|---|---|---|---|---|
| 1 | Phase I Study of HRS-4642 in Patients With Advanced Solid Tumors Harboring KRAS G12D Mutation | Recruiting | No Results Available | •Advanced KRAS G12D Mutant Solid Tumors | •Drug: HRS-4642 |
| 2 | Bortezomib in KRAS-Mutant Non-Small Cell Lung Cancer in Never Smokers or Those With KRAS G12D | Completed | Has Results | •Non-Small Cell Lung Cancer | •Drug: Bortezomib<br>•Drug: Acyclovir |
| 3 | A Study of ASP3082 in Adults With Previously Treated Solid Tumors | Recruiting | No Results Available | •Solid Tumor | •Drug: ASP3082<br>•Drug: Cetuximab |
| 4 | Phase II Trial of Vemurafenib and Sorafenib in Pancreatic Cancer | Recruiting | No Results Available | •Pancreas Cancer | •Drug: Vemurafenib<br>•Drug: Sorafenib |
| 5 | Combination of CAR-DC Vaccine and PD-1 Antibody in Local Advanced/Metastatic Solid Tumors | Recruiting | No Results Available | •Solid Tumor, Adult<br>•EphA2 Overexpression<br>•KRAS G12V<br>•KRAS G12C<br>•KRAS G12D | •Biological: KRAS-EphA-2-CAR-DC<br>•Drug: Abraxane<br>•Drug: Cyclophosphamide<br>•Drug: PD-1 antibody |
| 6 | A Study of ELI-002 in Subjects With KRAS Mutated Pancreatic Ductal Adenocarcinoma (PDAC) and Other Solid Tumors | Recruiting | No Results Available | •Minimal Residual Disease<br>•KRAS G12D<br>•KRAS G12R<br>•NRAS G12D<br>•NRAS G12R<br>•Pancreatic Ductal Adenocarcinoma<br>•Colorectal Cancer<br>•Non-small Cell Lung Cancer<br>•Ovarian Cancer | •Drug: ELI-002 2P |

**Table 5:** Ongoing clinical trials targeting KRAS G12D

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## Conclusions

Herein is analysed the detailed function of the KRAS protein, the signalling cascade, the biochemical changes when mutations occur, the frequency of the mutations and their proportion in colorectal cancer, as well as the therapeutic molecules targeting KRAS G12D that are under development. Association between CRC cases and KRAS mutation has been established several years ago but inhibition of the specific pathway has been particularly challenging. In the era of precision medicine, it is mandatory to focus on these mutations to understand their function and find a way to impede them. This could bring promising results in the treatment of mCRC.

*Georgios Chlorakis, March 2023*

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