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Review

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Research progress on *KRAS* mutations in colorectal cancer

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Abstract

The *RAS* gene family, responsible for signal transduction within the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K) pathways, is frequently involved in carcinogenesis, and alterations in its member genes can be detected, with variable frequency, in a wide variety of solid and hematological cancers. These alterations may behave as prognostic-predictive biomarkers and driver mutations, making them an interesting therapeutic target. Since their discovery, many strategies have been pursued to act on their signaling pathways. Indeed, in clinical practice, *KRAS*, the most prominent member of the *RAS* gene family, represents an especially elusive target in most malignancies; pathway inhibition is carried out upstream, on the EGFR receptor, or downstream, most frequently on the BRAF/MEK/ERK cascade. Recently, clinically relevant direct RAS inhibition has been successfully achieved with the development of potent and selective covalent inhibitors of *KRAS* c.34G>T (p.G12C). These latest-generation drugs represent both a new and interesting tool in the therapeutic armamentarium and a symbolic end to the myth of KRAS undruggability. However, their clinical relevance and appropriate place in treatment strategies remain to be determined.

Keywords: KRAS, targeted therapies, EGFR pathway, MAPK, colorectal cancer

INTRODUCTION

Identification of the *RAS* gene family can be traced back to the 1970s, with the definition of the oncogenic sequences of the Kirsten and Harvey strains of murine sarcoma virus[1]. The human homologs of these genes, however, were not identified until the 1980s; *HRAS* and *KRAS* are homologs to the Harvey and Kirsten murine oncogenes, respectively, while *NRAS* was identified in a human neuroblastoma cell line[2,3]. These genes encode for closely related GTPase proteins responsible for signal transduction within two main pathways, the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase pathways, involved in the regulation of cell growth, survival, and differentiation. In the scope of this review, we mainly focus on *KRAS*.

ROLE OF *KRAS* IN COLORECTAL CANCER CARCINOGENESIS

Cancer is the result of the gradual accumulation of gene mutations that lead to an increase of cell proliferation[4-6]. The evolution from benign to malignant lesions and their acquisition over time has been extensively studied, especially in colorectal cancer (CRC)[7]. Indeed, the so-called "Vogelgram", the most important model of cancer development, has been proposed in this tumor type. This model associates specific gene alterations to different stages of CRC development and identifies 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[7-9]. The gatekeeper event is represented by point mutations that inactivate the *APC* gene in early phases (at the stage of adenoma), while those occurring in the *TP53* gene are typically associated with late stages (carcinoma) or metastatic spreading[7,10].

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[7]. Overall, *KRAS* mutations provide only a small selective growth advantage to the cell, equal to a 0.4% increase in the difference between cell birth and cell death. Over many years, however, this slight increase can result in the development of a large *KRAS* mutant mass[6,7,9].

KRAS is regulated by tyrosine kinase receptors and, in turn, activates the MAPK signaling pathway that is mainly involved in determining cell survival, leading to a strong promotion of cell growth and replication. In particular, mutations in *KRAS* gene confer to cancer cells the ability to grow in lower glucose concentrations than those required for the growth of normal cells or cancer cells that do not have mutations in these genes[11,12].

A derivative of the "Vogelgram" is the involvement of *KRAS* in the phenomenon known as "chromosomal instability" (CIN), which is also considered to be the classical mechanism of CRC development since it represents the cause of up to 80%-85% of all CRC cases. CIN is characterized by imbalances in the number of chromosomes, thus leading to aneuploydic tumors and loss of heterozygosity[13].

MUTATION RATES IN A POPULATION-BASED COHORT OF COLORECTAL CANCER

KRAS mutation rates are different among CRC patients belonging to different ethnicities. 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%[14,15]. Most *KRAS* mutations in CRC occur within *KRAS* exon 2, with prevalence ranging from 10% to 47%, typically in codons 12 and 13. In addition, approximately 10% of patients with CRC are characterized by other types of *KRAS* mutations, in exon 3 (in codons 59 and 61, accounting for about 2%-3% of CRC) or exon 4 (in codons 117 and 146, with a mutation prevalence equal to 3%-4%). In addition, the *NRAS* brother gene is also frequently altered, in a mutually exclusive manner with respect to *KRAS*

mutations. *NRAS* is mutated in the same codons of *KRAS* (therefore, these positions are true hot-spot mutations), especially in exon 2 (observed in approximately 3%-5% of CRC) and exon 3 (2%-6% of CRC), while mutations in exon 4 are substantially absent[16,17].

KRAS in Caucasians

In Europe, the most relevant *KRAS* exon 2 mutations in CRC are c.35G>A (p.G12D) (37%), c.35G>T (p.G12V) (28%), and c.38G>A (p.G13D) (15%) changes[18]. However, there are important differences among European countries. For example, in Russia, there is a higher prevalence of c.35G>T (p.G12V) mutations (32.7%) and a lower one of c.38G>A (p.G13D) mutations (12.5%) in comparison with patients from other parts of Europe. Furthermore, in a geographically distinct region of Italy, Sardinia, mutation frequencies indicate a lower occurrence of the c.35G>A (p.G12D) change and a more frequent detection of other mutations in codon 12 [c.35G>A (p.G12D), 26%; c.35G>T (p.G12V), 23%; c.38G>A (p.G13D), 16%; c.35G>C (p.G12A), 7%; c.34G>T (p.G12C), 6%; c.34G>A (p.G12S), 3%; c.37G>T (p.G13C), 2%; c.34G>C (p.G12R), 2%; and c.38G>C/c.37G>A/c.38G>T (p.G13A/p.G13S/p.G13V), 2%][19]. These differences should be taken into account when assessing the response of CRC patients on chemotherapy and targeted therapy[20]. Concerning exons 3 and 4, the situation seems to be uniform across European countries, with the most common alterations [c.183A>C (p.Q61H), c.182A>T (p.Q61L), and c.182A>G (p.Q61R)] in exon 3 accounting for 3% of the totality of *KRAS* mutant cases and the alterations in exon 4 [mainly c.436G>C (p.A146P), c.436G>A (p.A146T), and c.437C>T (p.A146V)] for 4% of *KRAS* mutations[19].

KRAS in Asians

Concerning Asian populations, *KRAS* mutations are detected in 35%-40% of CRC cases in Chinese patients. In China, the mutations occur more commonly in codon 12 (79.1%) and codon 13 (20.4%) in exon 2, with c.35G>A (p.G12D) (37.8%), c.35G>T (p.G12V) (20.0%), and c.38G>A (p.G13D) (19.6%) changes as the most frequent mutation types. These three point mutations account for 77.3% (174/225) of *KRAS* gene mutations[21,22]. As a consequence, exons 3 and 4 mutations are less frequently detected, representing, 1%-2% and 4%-7% of the CRC population, respectively[23,24]. In particular, the most common mutations in exons 3 are c.183A>C (p.Q61H), c.182A>T (p.Q61L), c.182A>G (p.Q61R), characterizing 1.19%, 0.74%, and 0.15% of the cases, respectively, while the most relevant mutations [c.436G>C (p.A146P), c.436G>A (p.A146T), c.437C>T (p.A146V), and c.351A>C (p.K117N)] represent less than 4% of patients[23,25].

In South Korea, the frequency of *KRAS* mutations is superimposable with that observed in China (37%), mainly affecting codon 12 (73.8%) and codon 13 (24%). More in detail, the most frequent amino acid change is c.35G>A (p.G12D), which accounts for 36.9% of *KRAS* mutations, followed by c.38G>A (p.G13D) (24.2%) and c.35G>T (p.G12V) (21.9%)[26]. Similarities between South Korea and China are also observed in mutation frequencies and type of mutations in *KRAS* exons 3 and 4[26].

KRAS in Africans

Among continents, the most relevant difference of *KRAS* mutation rate in CRC has been observed in Africa. In Nigeria, the occurrence of *KRAS* exon 2 mutations (codons 12 and 13) has been described in only 21% of patients, meaning roughly half the rate of Caucasians and Asians[27]. Specific data concerning *KRAS* exon 3 and 4 mutations in the African population are still lacking.

KRAS differences in the heterogeneous American population

Another relevant difference in *KRAS* mutation rates has been observed in the comparison between African Americans and Caucasian Americans. *KRAS* mutations occurred significantly more frequently in African Americans than Caucasians (37% *vs.* 21%, $P = 0.01$) and were associated with worse stage or grade of CRC[28]. It is noteworthy that such a difference is mainly caused by codon 12 mutations: indeed, many

studies have reported that codon 12 mutations are significantly more frequent in African Americans than Caucasians ($P = 0.04$), whereas the frequencies of mutations in codon 13, exon 3, and exon 4 did not differ significantly according to ethnicity[27-29]. Interestingly, even within the same ethnic group, there can be differences. Namely, a higher rate of *KRAS* mutations has been reported in the Hispanic group (59%) compared to the general Caucasian population (38%)[15]. In addition, another relevant variability has been observed in Mexico: the *KRAS* mutation rate varies from 40% in the North Pacific cities to 59% in the central Mexican region[30]. By comparing mutation rates between Africa and African Americans and among the different ethnicities in the United States, it is clear that, more than race (which is, for instance, important for the occurrence of *EGFR* mutations in lung adenocarcinomas), the aspects that seem to play a prominent role in the occurrence of *KRAS* mutations in CRC are environment and diet. In the United States, the dietary lifestyle of the less wealthy populations is often characterized by junk food meals and lower fiber and fruit intake. Compared to African populations, African Americans definitely have an excessive intake of calories and a higher consumption of red and processed meat.

FREQUENCIES OF *KRAS* IN CRC AND OTHER CANCERS

Mutations involving *RAS* genes are the most frequent oncogenic alterations in human cancers [Table 1][31]. In particular, in the *RAS* family, *KRAS* is the most frequently mutated gene, followed by *NRAS* [Table 2][31]. Overall, *KRAS* is altered in 15.95% of cancers, with pancreatic, lung, colon adenocarcinoma, colorectal, and rectal adenocarcinomas having the greatest prevalence of alterations[17]. In addition to CRC, *KRAS* oncogene activation through point mutations represents the main oncogene alteration in pancreatic cancer, where such an alteration is observed in more than 80% of cases. A percentage of alteration close to the one observed in CRC is found in lung adenocarcinomas and cholangiocarcinoma[32,33][Table 3].

The *KRAS* mutation pattern is tumor specific [Table 3][17]. As mentioned above, in CRC, *KRAS* exon 2 mutations occur at an average rate of nearly 40%; in particular, c.35G>A (p.G12D) is detected in 37% of cases; c.35G>T (p.G12V) in 29%; c.34G>T (p.G12C) in 7%; c.35G>C (p.G12A) in 9%; c.34G>A (p.G12S) in 7%; c.38G>A (p.G13D) in 14%; and c.34G>C (p.G12R) in less than 1%. Interestingly, in CRC, the most prevalent *KRAS* exon 2 mutations, c.35G>A (p.G12D) and c.35G>T (p.G12V), are weak drivers, whereas *BRAF* c.1799T>A (p.V600E) is a pivotal driver[18,34]. Compared to CRC, recent data define higher rates of *KRAS* exon 2 mutations in pancreatic tumors (79.7%) and lower frequencies in non-small cell lung cancer (NSCLC) (27.1%)[17] [Table 3]. In particular, c.35G>A (p.G12D) mutation incidence is the highest in pancreatic cancer and the lowest in lung adenocarcinoma and c.35G>T (p.G12V) mutation is the most frequent in ovarian cancer and the least in cholangiocarcinoma. The most peculiar *KRAS* mutation is c.34G>T (p.G12C), which is by far the most frequent in lung adenocarcinoma (approximately 40%) and significantly less frequent in other cancers (no more than 10%). The other forms of mutation in exon 2 are much less prevalent but again related to specific cancer types: the c.35G>C (p.G12A) mutation characterizes endometrial cancer, the c.34G>A (p.G12S) mutation is cholangiocarcinoma specific, and the c.34G>C (p.G12R) mutation is typical of pancreatic cancer, while the c.38G>A (p.G13D) mutation is strictly associated with CRC, suggesting unique carcinogenic effects[18]. These mutational patterns are caused by various carcinogens and/or mutagenic processes, such as DNA repair deficiencies, and therefore are unique signatures of various cancer types[35]. For this reason, the result of the processing of red meat, producing O6-methylguanine and heterocyclic aromatic amines compounds that produce bulky DNA adducts, which oftentimes cannot be removed from DNA due to nucleotide excision repair system errors, can be a critical issue. This situation can bring to the occurrence of *KRAS* (and *APC*) mutations in CRC[36]. Furthermore, it has been demonstrated that the c.34G>T (p.G12C) change occurs predominantly in women affected by CRC upon exposure to acrylamide metabolites present in heat treated carbohydrate-rich foods[37].

Table 1. *RAS* mutations percentage detected in different types of cancers

Cancer	*RAS* mutations percentage
Pancreatic Ductal Adenocarcinoma	96%
CRC	52%
Multiple myeloma	43%
Lung adenocarcinoma	32%
Skin cutaneous melanoma	29%

CRC: Colorectal cancer (Hobbs *et al.*[31], 2016).

Table 2. Specific *KRAS*, *NRAS*, and *HRAS* mutation percentages detected in different types of cancer

Cancer	Rate of *KRAS* in the total of RAS mutations	Rate of *NRAS* in the total of *RAS* mutations	Rate of *HRAS* in the total of RAS mutations
Pancreatic ductal adenocarcinoma	100%	0%	0%
CRC	86%	14%	0%
Multiple myeloma	55%	45%	0%
Lung adenocarcinoma	96%	3%	1%
Skin cutaneous melanoma	94%	3%	3%

CRC: Colorectal cancer (Hobbs *et al.*[31], 2016).

Table 3. Rates of *KRAS* mutations in three different types of cancer (pancreatic cancer, CRC and NSCLC)

Cancer	Mutation rates			
	KRAS	*KRAS* exon 2	*KRAS* exon 3	*KRAS* exon 4
Pancreatic	86.3%	79.7%	6.6%	0.16%
CRC	44.2%	37.6%	2.63%	3.7%
NSCLC	29.6%	27.1%	1.89%	0.24%

CRC: Colorectal cancer; NSCLC: non-small cell lung cancer (AACR Project GENIE Consortium, 2017).

As far as *KRAS* exons 3 and 4 is concerned, the results obtained by the AACR Project GENIE Consortium describe that these mutations are significantly rarer with respect to those occurring in exon 2; indeed, they are detected in 1.15% and 0.62% of cancer patients, respectively[17]. More in detail, the mutations in exon 3 characterize 2.63% of CRC and 1.89% of NSCLC, whereas they are more diffused in pancreatic adenocarcinoma patients (6.6%)[17] [Table 3]. A different trend is observed for exon 4: these mutations are more frequent in CRC (3.7%) compared to pancreatic adenocarcinoma (0.16%) or NSCLC (0.24%)[17] [Table 3].

Differences among cancer types are also observed concerning the different types of mutation: the c.182A>G (p.Q61R) change is very frequent in pancreatic adenocarcinoma (8.33%), at odds with lung adenocarcinoma (0.06%) or CRC (0.31%). The other two most common exon 3 mutations, the c.183A>C (p.Q61H) and c.182A>T (p.Q61L) changes, are not relevant in pancreatic cancers (less than 0.1%) but are detected in 1.2% and 0.4% of NSCLC and 0.98% and 0.36% of CRC, respectively[17].

Mutations in *KRAS* exon 4 have been registered by the AACR Project GENIE Consortium at lower frequencies compared to alterations in exon 3, with the c.436G>C (p.A146P) change detected only in CRC (0.07%) and not in NSCLC or pancreatic cancer. On the contrary, the c.436G>A (p.A146T) and c.437C>T

(p.A146V) mutations are still more frequent in CRC (2.42% and 0.6%, respectively) but can be found in NSCLC as well (0.07% and 0.05%, respectively)[17].

Correlation between KRAS and sidedness

In addition to ethnic differences, the mutational status of *KRAS* can be different in CRC on the basis of tumor location. In particular, these differences have been investigated comparing right-sided (RCC) *vs.* left-sided colon cancers (LCC). Often grouped as one disease, RCC (including cancers arisen in cecum, ascending colon, and hepatic flexure) and LCC (with cancers developed in the splenic flexure, descending colon, and sigmoid colon) actually represent clinically and molecularly distinct entities with significant differences in their prognosis and treatment outcomes.

The molecular differences between RCC and LCC have recently been extensively investigated[38]. This study describes significant differences in mutational profiles between RCC and LCC and it demonstrates that the early common somatic gene mutations are associated with the selection of different subsequent genomic events in RCC compared to LCC[38]. Focusing the attention on *KRAS*, Salem *et al.*[39] demonstrated that *KRAS* mutations are detected in 61%-71% of RCC and at a lower frequency in LCC (30%-36%), data also confirmed by another independent study.

In addition, these differences were also described by other investigators who included cohorts of patients from different ethnicities. In Caucasian cohorts, *KRAS* mutations were detected in 52%-65% of RCC and 35%-43% of LCC while, in Asian populations, in 30%-35% and 20%-25%, respectively[27,40,41].

Furthermore, the same differences between *KRAS* mutation rates in LCC and RCC have been confirmed in tumors developed in adolescents and young adults (age lower than 50 years old). In this age-selected population, next-generation sequencing analyses showed higher *KRAS* mutations rates in RCC than LCC (respectively, 64.1% *vs.* 45.5%, $P = 0.035$)[39]. These results may explain different responses obtained to anti-EGFR therapies. Indeed, LCC patients demonstrate a longer overall survival (OS) than individuals affected by RCC.

Another stratification based on tumor location has been performed by comparing cancers arising in the colon *vs.* the rectum. In rectal cancers, *KRAS* alterations are observed in nearly 42% of patients, a value comparable to that of LCC and lower if compared to RCC[38,39]. Similar data have been obtained by investigations on Asian populations with younger ages[27,39]. All the aforementioned data have been obtained by the analysis of *KRAS* exon 2; data from exons 3 and 4 are still lacking.

CLINICAL RELEVANCE AND PROGNOSTIC/PREDICTIVE VALUE OF *RAS* GENE FAMILY ALTERATIONS

Mutations in the *KRAS* gene are detected in approximately 25% of all human cancers; rates are highest in pancreatic carcinoma, reaching above 80%, while, in colorectal cancer, they are found in about 40% of cases. *NRAS* mutations are less frequent, appearing in about 7% of cases. Their role in carcinogenesis has been proven in the preclinical setting, and, despite not being usually sufficient to initiate cancer growth and progression, they contribute to the development and progression of both adenomas and adenocarcinomas[42]. *KRAS* mutation prevalence reaches 30% in lung adenocarcinomas and 5% in lung squamous cell carcinomas, and it has been shown to vary between 45%-54% in extrahepatic and 10%-15% in intrahepatic cholangiocarcinomas. They can also be detected, with variable frequency, in a wide variety of other solid and hematological cancers[43].

Several studies have explored a possible prognostic role of *KRAS* mutations; however, results are not uniform across experiences, tumor types, and stages. On the other hand, *KRAS* mutations seem to play a role as predictive markers of treatment response, or lack thereof, in specific clinical settings. Most notably in CRC, *KRAS* mutations predict a lack of response to EGFR-directed treatments.

The prognostic role of *KRAS* remains unclear, especially for non-metastatic stage II and III CRC, and it is controversial in both microsatellite stable (MSS) and microsatellite unstable (MSI) CRC[44]. Some studies have reported a possible association between *KRAS* mutations and survival, but data on this topic are quite controversial.

Among the studies suggestive of *KRAS* as a prognostic marker, one in particular showed a high prevalence of *KRAS* (and *BRAF*) in stage II and III MSI colon cancer and, when combined, their association with poorer survival compared to cancers wild-type (wt) in both these genes. The 5-year cancer specific survival (CSS) was 93% (95%CI: 84%-100%) for patients characterized by both *KRAS* and *BRAF* wild-type genes, whereas it was 76% (95%CI: 67%-85%) in patients with at least one mutation in *KRAS* or *BRAF*[45].

Eklöf *et al.*[46] and Nash *et al.*[47] proposed a prognostic role of *KRAS* mutations in MSS patients. Stage I and II patients with *KRAS* mutations have a high mortality, with a 5-year survival of only 55% in *KRAS* mutant MSS cases *vs.* 68% in *KRAS* wt MSS cases. The correlation between *KRAS* mutations and survival in MSS cases has not been observed in stages III and IV, where only a strong trend towards worse survival has been described[47].

Beside OS and CSS parameters, disease-free survival (DFS) has been investigated. Sinicrope *et al.*[48] reported, in a cohort of CRC not subdivided on the basis of microsatellite status, that *KRAS* mutations are statistically associated with poorer DFS (HR = 1.44; 95%CI: 1.21-1.70; $P < 0.001$).

Recently, Marco *et al.*[49] explored the relationship between *KRAS* mutation and immune-related changes in microenvironment in CRC; *KRAS* mutant tumors were enriched of M2 macrophages, Treg lymphocytes, and IL-17, deemed as pro-tumorigenic immune markers, and had fewer anti-tumorigenic CD4 T-helper lymphocytes, compared to *KRAS* wild-type ones.

While many studies have a propensity to suggest *KRAS* mutations to influence survival outcome in CRC patients, others failed to confirm such an association. Roth *et al.*[50] described that *KRAS* molecular status does not have any prognostic value regarding OS (HR = 1.05; 95%CI: 0.85-1.28; $P = 0.66$) and relapse-free survival (HR = 4.99; 95%CI: 0.65-3.91; $P = 0.31$). In addition, this association was not statistically relevant even when patients were stratified by stages or microsatellite status. These findings were also confirmed by other studies[51-56]. Interestingly, when *KRAS* mutational status was stratified with respect to the type of mutation, patients with c.38G>A (p.G13D) may show significantly shorter survival rate than those with wild-type genotype[56]. Overall, the true role of *KRAS* mutational status in CRC survival is still debated, and most of the scientific literature seems against its prognostic role.

RAS in metastatic colorectal cancer

EGFR-directed antibodies, namely cetuximab and panitumumab, are routinely employed in the setting of first and further line therapy for metastatic colorectal cancer, in combination with standard chemotherapy. However, EGFR membrane expression evaluated by immunohistochemistry does not correlate with treatment benefit[57]. Rather, alterations in the signaling pathways downstream of EGFR may override the therapeutic effect of EGFR inhibition; driver mutations in the *RAS* gene family represent one such instance

and can lead to a detrimental effect from EGFR-directed therapies[58]. Consequently, confirmation of *RAS* wild-type status is mandatory before the start of such treatments. Mutations most commonly occur in exon 2 (codons 12 and 13) and are less frequent in exons 3 (codons 59 and 61) and 4 (codons 117 and 146). However, current guidelines recommend that extended *RAS* testing exploring exons 2, 3 and 4 (and, thus, codons 12, 13, 59, 61, 117, and 146) should be carried out, and that only patients that show pan-*RAS* wild-type status may be treated with anti-EGFR agents[59]. In accordance with NCCN guidelines, testing may be carried out on histological or cytological samples from either the primitive lesion or a metastasis[60]. Available platforms range 85%-100% sensitivity and 98%-100% specificity[42]. *RAS* mutation is considered an early event in carcinogenesis, and consequently the mutational status is usually concordant between the primary tumor and metastatic lesions[61]. More recently, liquid biopsy has been proposed as an alternative testing strategy. With appropriate testing methodologies such as BEAMing-PCR, excellent results have been obtained, with 90.4% positive agreement, 93.5% negative agreement, and 91.8% concordance with tissue-based methods[62,63].

As stated above, tumor sidedness (origin of the primary tumor on the right- *vs.* left-side of the colon) also appears to play a predictive role, with right-sided tumors deriving scarce benefit from anti-EGFR treatment in the first line, despite wild-type *RAS* status[64]. The different biology of right- *vs.* left-sided tumors is reflected in the 2014 consensus molecular subtypes classification, which is based on gene expression profiling and identifies four subgroups: MSI immune, canonical, metabolic, and mesenchymal. Right-sided tumors present characteristics of either the MSI immune or metabolic subtype[65].

Colorectal tumors that display pan-*RAS* wild-type status at diagnosis may acquire *RAS* mutations over time and the course of treatment; indeed, selective pressure from EGFR-directed therapies may favor pre-existing or newly appearing *RAS* mutant clones that are not sensitive to EGFR targeting. Another possible mechanism of resistance lies in the appearance of mutations in the extracellular domain of EGFR, hindering the binding of the therapeutic agent to the receptor[66].

Upon progression to a treatment line including an EGFR targeting agent, current practice dictates a change in the chemotherapy backbone, the biological agent, or both. Once a patient shows signs of progressive disease to anti-EGFR-directed treatment, current guidelines and most regulatory agencies do not consider the option of a rechallenge, as it is expected that acquired resistance will not be reversible. However, recent data have challenged this, highlighting the role of clonal evolution in shaping tumor responsiveness to targeted agents. Goldberg *et al.*[66] demonstrated that *RAS* mutant clones can emerge in originally *RAS* wild-type CRC over the course of anti-EGFR treatment, leading to acquired resistance[66,67].

The CRICKET trial enrolled patients who had successfully been treated with cetuximab plus chemotherapy for a duration of at least 6 months; upon progression, they were treated with bevacizumab-containing regimens. Those who experienced benefit from this second line for a duration of at least 4 months, and then progressed, received cetuximab in combination with irinotecan as a rechallenge strategy[68]. Four out of 27 patients (14.3%) obtained a partial response (PR), and the disease control rate was 53.5%. Median PFS and OS were 3.4 and 9.8 months, respectively. *RAS* mutational status was assessed by liquid biopsy before the start of the cetuximab rechallenge; *RAS* mutations were found in 48% of evaluable patients, and all 4 patients who obtained PR were identified as *RAS* wild-type. Median reported PFS was 4 months for *RAS* wild-type patients *vs.* 1.9 months for *RAS*-mutant ones; median OS was 12.5 months *vs.* 5.2 months. These results suggest that the analysis of *RAS* status among treatment lines might help in predicting response to anti-EGFR rechallenge[68].

In a similar small proof-of-concept study by Gazzaniga *et al.*[69], 11 patients with *RAS*-mutated metastatic CRC who had been treated with bevacizumab-containing regimens were enrolled, and *RAS* status was tested upon progression by liquid biopsy, employing the plasma-based Idylla™ system. Five out of 11 patients tested negative; 4 of them obtained confirmation of *RAS* wild-type status by Ion Torrent-Personal Genome Machine and were treated with anti-EGFR based therapies with longitudinal circulating tumor DNA (ctDNA) monitoring. The PFS of these patients was 12, 10, and 6 months for the three that were treated in the second line and 4 months in a patient treated in the fourth line.

Overall, this study suggests that antiangiogenetic agents may be able to revert *RAS*-mutated tumors to wild-type status, most likely through partially selective killing of *RAS*-mutant tumor cells. The authors hypothesized that *RAS*-mutated cells may be more sensitive to alterations in the vascular supply secondary to antiangiogenetic treatment. Additionally, Gazzaniga *et al.*[70] assessed the *RAS* status concordance between tissue samples and plasma in 20 patients with *RAS*-mutant tumors; 15 patients with concordant results were then monitored by liquid biopsy over the course of antiangiogenetic treatment with the Idylla™ platform. Eleven out of 15 concordant patients (73%) switched to *RAS* wild-type after the first line. Five of them went on to receive EGFR inhibitors in the second line setting, with a significant clinical benefit, supporting the hypothesis that anti-EGFR treatment may be of benefit to originally *RAS*-mutated tumors that revert to wild-type status upon treatment with antiangiogenic agents, in a similar way as in patients in the CRICKET trial upon rechallenge after reversion.

Rates of *RAS* mutation loss may be as low as 1.6%-8.8%, in contrast with previous reports reaching as high as 20%, as demonstrated by a study conducted by MD Anderson Cancer Center[71]. Moreover, such rates may be influenced by the sensitivity of the analytic method, particularly when liquid biopsy is employed[71]. Plasma-based methods have shown promising results in *RAS* mutation testing in CRC, with an overall agreement between matched tumor and ctDNA samples of over 90%, 89%-96% sensitivity, and 90%-100% specificity in many retrospective series. However, when highly sensitive tissue-based methods are used, the concordance rate can drop to 78%-88%, with 83%-91% sensitivity and 70%-85% specificity[72], although some studies have suggested that the detection of underrepresented clones by such techniques does not carry clinical significance[73]. Among clinicopathological variables that are known to influence the abundance of ctDNA and, consequently, the reliability of results are cancer burden, stage, tumor biology (e.g., apoptosis rate and metastatic potential), and vascularization[72,74-76]. It cannot be excluded that the use of antiangiogenetics, beside contributing to a response that possibly decrease the overall tumor burden, may also impact the remodeling of the tumor vasculature that could potentially influence tumor DNA access to systemic circulation. Some of the limitations of liquid biopsies are likely to be overcome in the near future, in part by preanalytical and analytical standardization and in part by identifying a threshold of clinical significance and the setting in which tissue- and plasma-based methods may be interchangeable. However, current data suggest that a measure of caution should still be applied.

The fluctuations in *RAS* mutation levels according to applied treatment are perhaps among the most evident examples of how the interplay between multiple genetic and environmental factors can influence disease course and evolution. Indeed, each individual tumor in each individual host has a unique pathogenetic history, shaped by the interactions between genetics, exposure to environmental and behavioral risk factors, immune response, applied treatments, and the microbiome; and this complex network of interactions can result in specific molecular and pathological peculiarities within a disease continuum[77,78].

It should be noted that the existence of this network adds a layer of complexity beyond the relatively simple identification of a clear and evident association between, e.g., a mutation in a specific gene and a resulting

prognostic phenotype or clinical outcome. Other significant instances that exemplify this complexity are represented by the identification of genetic alterations that interact with lifestyle factors of pharmacological interventions: a study by Song *et al.*[79] suggested that single nucleotide polymorphism (SNP) rs4444235 at 14q22.2 and SNP rs2423279 at 20p12.3 appear to modify the benefit from regular exercise and regular aspirin use, respectively, in terms of risk of developing CRC. Similarly, a study by Cook *et al.*[80] has shown that each *KRAS* allele is associated with a distinct, tissue-specific comutation network, and, moreover, these associations are tissue-specific. The study, classification, and interpretation of each disease's unique peculiarities, as well as the definition of patterns and causal relationships, is likely to benefit from the systematic application of "big data" analysis methodologies, within the paradigm defined as molecular pathological epidemiology[77,78].

RAS as a therapeutic target

The frequent occurrence of *RAS* mutations in human cancers, along with the relevance of its role either as a driver mutation [as is the case for *KRAS* c.34G>T (p.G12C)] or in the development of treatment resistance as exemplified by colorectal cancer, makes it an attractive target for drug development in principle. Multiple approaches have been attempted in order to obtain clinically relevant inhibition of KRAS; however, most strategies until recently have been plagued by unsuccess[81,82].

Direct RAS inhibition was first attempted by identifying molecules that could preferentially bind the RAS-GTP pocket. This approach was unsuccessful, possibly due to the extremely high affinity between RAS and GTP[83]. Consequently, alternative targeting strategies have been explored.

Inhibition of RAS protein expression by employing antisense oligonucleotides was successfully explored in the preclinical setting. Ross *et al.*[84] tested the compound AZD4785 in mouse models of NSCLC and primates, showing marked selective depletion of KRAS protein with downstream inhibition, and in the absence of feedback activation of the MAPK pathway, which had been observed with other targeting strategies.

Similarly, Gray *et al.*[85] attempted HRAS targeting by antisense oligonucleotides (ISIS 2503) in *HRAS* mutant cells and obtained a 90% reduction in protein expression; clinical testing followed in the setting of phase I and II studies, including one involving CRC patients and one involving pancreatic cancer patients; however, clinical activity was globally unsatisfactory and development was not pursued further[85-89].

An alternative strategy involves the inhibition of post-translational modifications, namely prenylation, characteristic of RAS molecules that are implied in membrane localization and activity. These modifications require the addition of either a farnesyl or geranylgeranyl group, catalyzed by specific enzymes. The compound tipifarnib is a farnesyl transferase inhibitor whose activity was tested in various phase II trials on patients with different solid tumors with unsatisfactory results; phase III trials were carried out in patients with advanced CRC and as a maintenance strategy in AML, however no survival benefit was detected in either setting[89-92].

Lonafarnib, another drug in the same class, showed insufficient activity in CRC, urothelial cancer, and NSCLC[93-95]. It has been suggested that the failure of this line of attack might be due to the fact that KRAS and NRAS can alternatively undergo either farnesylation or geranylgeranylation, thus negating benefit from the inhibition of a single pathway. The discovery that HRAS, on the other hand, is solely dependent on farnesylation has led to the expectation that farnesyl transferase inhibitors should be active in patients with *HRAS* mutant cancers, and initial results from a phase II trial testing this strategy are encouraging[96]. At the

same time, a geranylgeranyl transferase inhibitor has been developed, but clinical testing revealed a lack of efficacy as monotherapy, and dual inhibition appears to be too toxic[97-99].

A third possible step in the prenylation process is represented by carboxymethylation, catalyzed by the enzyme isoprenylcysteine carboxylmethyltransferase (ICMT); in this regard, ICMT inhibition is being investigated as a therapeutic strategy[100-103].

Due to these difficulties, efforts were made to target signaling downstream of RAS, through the inhibition of its effectors RAF, MEK, and ERK. Targeting of this cascade has proven a complex endeavor.

BRAF inhibition was first attempted with sorafenib, a tyrosine kinase inhibitor, and the drug has entered the therapeutic armamentarium against different malignancies, including hepatocellular carcinoma, renal cell carcinoma, gastrointestinal stromal tumors, and thyroid carcinoma[104-106]. Nevertheless, sorafenib is not considered a specific or potent BRAF inhibitor, and it has been demonstrated that its activity is likely due to multikinase inhibition[107].

Second generation BRAF inhibitors, such as vemurafenib, dabrafenib, and encorafenib, have shown relevant clinical activity, either alone or in combination with MEK inhibitors, particularly in cancers carrying the c.1799T>A (p.V600E) mutation; most notably, such a strategy is routinely applied in melanoma, but has also shown favorable results in other settings[108-110]. Recently, results from the BEACON trial have shown that the combination of encorafenib with the anti-EGFR inhibitor cetuximab, with or without the addition of the MEK inhibitor binimetinib, is effective in *BRAF* c.1799T>A (p.V600E) mutant CRC with a survival advantage over irinotecan-based chemotherapy plus cetuximab (9.3 months *vs.* 5.9 months)[111].

However, several issues remain unsolved. Mechanistically, it was expected that BRAF inhibitors could prove effective in *BRAF* wild-type, *KRAS* mutant cancers, given its downstream localization in the pathway; however, it has been observed that this strategy leads to a paradoxical increase of ERK signaling activity. This can be explained by taking into account that RAF proteins tend to dimerize, and binding of an inhibitor to one protomer induces a conformational change resulting in activation of the other, in a process defined as negative cooperativity - thus, for instance, a BRAF/CRAF heterodimer can increase ERK signaling through CRAF activation[112-114]. This plays a pivotal role in the development of secondary malignancies, which represent a known side effect of BRAF inhibitors, and it is also relevant in drug resistance that generally ensues over the course of treatment. Indeed, paradoxical ERK activation can also occur in *BRAF* mutant cancers. An excessive ERK signaling is usually lethal to cells, thus prolonged drug exposure can favor the emergence of BRAF-inhibition addicted cell clones with low intrinsic ERK signaling that require the paradoxical ERK activation for growth and can be responsive to a treatment holiday[115,116].

To date, two strategies are being explored to address this issue by impeding hetero- or homodimerization: the development of pan-RAF inhibitors, which can overcome CRAF-mediated resistance by binding both protomers, and the so-called "paradox breakers", which induce a conformational change in the RAF dimer interface[112]. However, another approach challenges bypassing the paradoxical effect by direct targeting of MEK and ERK. While some MEK inhibitors (binimetinib, cobimetinib, and trametinib) are already employed in the treatment of *BRAF* mutated cancers, ERK inhibitors, on the other hand, have not yet transitioned into clinical practice and suffer from the hindrance of a lower therapeutic index, due to significant inhibition of the RAS/RAF/MEK/ERK pathway in healthy cells[117].

More recently, a direct approach to RAS targeting has come back to the forefront, as covalent inhibitors were developed targeting specific *RAS* mutations. The covalent inhibitor sotorasib, specifically targeted at the *KRAS* c.34G>T (p.G12C) mutation, is able to bind KRAS and lock it in an inactive state, thus suppressing downstream ERK phosphorylation. After favorable preclinical results, a phase I trial (CodeBreaK 100) was carried out in 129 patients with heavily pretreated solid tumors harboring the *KRAS* c.34G>T (p.G12C) mutation. The response rate was 32.2% in NSCLC patients, with 88.1% disease control rate and a median progression-free survival (mPFS) of 6.3 months. Specifically, in CRC patients, the response rate was 7.1% and DCR was 73.8% with a mPFS of 4 months, suggesting that *KRAS* c.34G>T (p.G12C) does not represent the only oncogenic driver in these patients. Indeed, Amodio *et al.*[118] compared in vitro response to sotorasib in CRC- and NSCLC-derived, c.34G>T (p.G12C) mutated cell lines. They observed that downstream ERK inhibition obtained with sotorasib monotherapy is not sustained in CRC. Additionally, CRC *in vitro* models, unlike NSCLC models, retain sensitivity to upstream growth factor stimulation mediated by receptor tyrosine kinases, mainly EGFR, which can interfere with c.34G>T (p.G12C) blockade. Based on these data, a dual inhibition strategy was explored, revealing that not only did cetuximab sensitize c.34G>T (p.G12C) CRC cell lines to sotorasib, but, vice versa, the same combination strategy also reverted c.34G>T (p.G12C)-mediated secondary resistance to anti-EGFR antibodies[118].

Within the CodeBreaK 100 trial, responses were also observed with sotorasib monotherapy in patients with pancreatic, endometrial, and appendiceal cancers and melanoma; and tolerance was good, with Grade 3 or 4 treatment-related toxicities occurring in 11.6% of patients[119]. Aside from the clinical relevance of these results, requiring corroboration in larger trials, these data were enthusiastically hailed as the first demonstration that RAS is, indeed, a druggable target. Nonetheless, the usefulness of sotorasib is limited to cancers harboring the c.34G>T (p.G12C) mutation. Similar to BRAF inhibitors, combination strategies are being investigated[120]. These may involve multitarget inhibition of the EGFR/RAS/RAF/MEK/ERK pathway, with the addition of an EGFR inhibitor, a MEK inhibitor, or both, or an Src homology containing protein tyrosine phosphatase 2 (SHP2) allosteric inhibitor; other approaches include associating mTOR or cyclin-dependent kinase targeting and immune checkpoint inhibition, with or without with the addition of cytotoxic chemotherapy.

Another recently developed covalent *KRAS* c.34G>T (p.G12C) inhibitor is adagrasib, whose activity is being investigated within the phase I/II KRYSTAL-1 trial. Interim results were presented in the form of abstract at the American Society of Clinical Oncology 2020 meeting[121]. In total, 110 patients with advanced solid tumors and a *KRAS* c.34G>T (p.G12C) mutation, who had already exhausted standard treatment lines, received treatment with adagrasib; similar to what was reported in the CodeBreaK 100 trial, the best results were observed in the 51 patients with a NSCLC diagnosis, who reached 45% objective response rate (ORR) and 96% disease control rate (DCR), while toxicity was evaluated on all participants, with only two instances of serious adverse events (namely, hyponatremia). Data concerning 18 participants with CRC were also presented, with 17% ORR and 94% DCR[122]. As with sotorasib, combination strategies associating adagrasib with other agents, such as checkpoint inhibitors, anti-EGFR antibodies or TKIs, or SHP-2 inhibitors, are being explored[123,124].

CONCLUSION

Alterations involving RAS proteins and their pathway play a significant role in the biology of various cancer subtypes, as oncogenic drivers, prognostic and predictive markers, and possible targets. The high prevalence of RAS mutations across cancer types makes it an interesting therapeutic target. However, RAS is situated at the center of a complex signaling network whose redundancy and interconnection with other pathways offer significant protection from most inhibition strategies. Several different approaches are being pursued

in the attempt to overcome these difficulties, either by targeting multiple proteins in the pathway or by combining RAS-directed treatments with different classes of agents, such as chemotherapy and immunotherapy, and more recently by direct targeting of specific *KRAS* mutations. The development of new therapeutic agents such as sotorasib and adagrasib possibly represents the first crack in the wall of KRAS undruggability. Although their purpose of application is limited to a specific mutation, and their precise role in the therapeutic landscape is yet to be defined, in terms of their relevance in specific cancer subtypes, usefulness as monotherapy or in one of many possible combinations, and magnitude of clinical benefit in a real-world setting. Moreover, the application of "big data" analysis techniques within the paradigm of precision medicine and molecular pathological epidemiology may improve our understanding of the role of *RAS* mutations in relation to other genetic and environmental factors, possibly paving the way to personalized management strategies in the setting of prevention, diagnosis, and treatment.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Cefalì M, Epistolio S, Palmarocchi MC, Frattini M, De Dosso S
Writing original draft: Cefali M, Epistolio S
Writing review & editing: Cefalì M, Epistolio S, Palmarocchi MC, Frattini M, De Dosso S
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Not applicable.

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