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Drug Combination Therapy for RAS-related Colorectal Cancer*

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Abstract RAS-related signaling system plays an important role in the occurrence and development of colorectal cancer, which is closely related to the proliferation, metastatic potential, and apoptosis of colorectal cancer cells. However, treating this type of cancer with a single medicine, either targeted therapies or chemotherapies, is not always the best option. In recent years, RAS-related signaling pathway inhibitors have been utilized in conjunction with other medications with promising outcomes in clinical trials and preclinical investigations. When used in concert with other anti-cancer drugs, EGFR inhibitors, VEGF inhibitors, RAS direct inhibitors, MEK inhibitors, and RAF inhibitors have shown particularly impressive performance. In terms of clinical value, combining cetuximab with chemotherapy regimens, EGFR inhibitors with chemotherapy regimens, and EGFR inhibitors with other anti-cancer drugs dramatically enhanced important indicators in patients with colorectal cancer who had wild-type RAS. However, treatment options for patients with RAS-mutant colorectal cancer have been less favorable, and in this context, anti-angiogenic and anti-EGFR agents and immune checkpoint inhibitors have been approved as second-line treatment options. In preclinical studies, inhibitors that directly target RAS have been shown to be effective in combination with other drugs, and other treatment options have also shown good results, giving patients with colorectal cancer unlimited hope. This review focuses on the relationship between RAS-related signaling pathways and colorectal cancer, combination tactics in clinical trials and preclinical studies, and research on drug resistance mechanisms linked with composition administration in order to lay the foundation for future clinical multidrug therapy strategies.

Key words RAS, colorectal cancer, signaling pathway, EGFR inhibitors, drug combinations, resistance
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Colorectal cancer (CRC) is the third greatest cause of death globally, accounting for 10% of new cancer cases and 9.4% of cancer deaths, according to revised projections for the global cancer burden in 2020 from the International Agency for Research on Cancer (IARC)[1]. Approximately half of people with CRC will acquire metastatic colorectal cancer (mCRC), and the median survival time after diagnosis is just 2-3 years[2]. It is now generally accepted that malignancies are genetic diseases caused by multiple genes and step alterations, as well as that an activated proto-oncogene has the potential to transform into an oncogene and initiate tumorigenesis. The protein expressed by the *RAS* gene family, a highly conserved proto-oncogene that includes *KRAS*, *NRAS*, and *HRAS*, is not only a membrane-bound guanosine triphosphate (GTP)/guanosine diphosphate (GDP) binding protein but also an essential part of the epidermal growth factor receptor (EGFR) signaling cascade [3-4]. *KRAS* gene mutations account for 35%-40% of CRC and are more likely to arise in the early stages of tumor growth, and they are also remarkably constant across primary and metastatic lesions. Given

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that therapy has no effect on the *KRAS* gene and that an activated *KRAS* gene may rapidly produce additional protein *via* translation and transcription, clonal cell proliferation and tumor formation are recognized as inevitable outcomes.

CRC may be caused by dietary or hereditary factors, however, the exact etiology is unknown. The molecular process of metastasis is not fully understood, and mCRC can exhibit a wide range of clinical characteristics and molecular pathways depending on where it spreads[5]. According to the report[6], CRC patients with abdominal metastases have a dismal prognosis even after chemo, and it has risen to the position of the second major cause of death in individuals with CRC. In other studies, over 80% of patients with localized CRC experienced a recurrence following surgical therapy, and about 50% relapsed because they had micro-metastases prior to surgery[7]. Moreover, chemotherapy has limited effectiveness and is less successful in treating CRC with distant metastases while being frequently used to treat CRC with regional metastases. Therefore, developing novel therapies is crucial. Numerous clinical trials have demonstrated that concomitant administration of various inhibitors that target RAS directly as well as their downstream and upstream targets, or their administration in combination with chemotherapeutic regimens, could be extraordinarily beneficial in the treatment of mCRC.

In the CRC, it should be highlighted that the therapeutic efficacy and importance of different RAS-related medication combinations or synergistic use with chemotherapeutic treatments have not been adequately explored. This study included a comprehensive analysis of multiple aspects in order to provide the framework for the synergy of RAS-related preparations in the treatment of CRC and to act as a resource for the development of potential combination therapies.

1 RAS−related pathways and CRC

CRC occurrence and progression are the result of a multistage, complicated process that involves intestinal epithelial cell differentiation and proliferation as well as further apoptosis and survival mechanisms[8]. In these pathological changes, an abnormal cell signal transduction network always runs through various stages of tumor formation and development. The most prominent among these cell signaling pathways include RAS-RAF-MAPK, PI3K-AKT-mTOR, Wnt-catenin, TGF-Smads, Jak-STAT, Hedgehog, and Notch. It has been shown that CRC associated with RAS pathway activation accounts for about 55%, of which KRAS and NRAS mutations occur in 45% of CRC[9]. And only 10%−20% of cases are eligible for resection owing to multifocal disease, insufficient functional liver reserve, and poor anatomical localization. Studying how cell signaling changes with RAS as the core throughout the malignant progression of CRC can disclose the process of disease progression and help scientists extract or synthesize novel medications that specific targets (Figure 1).

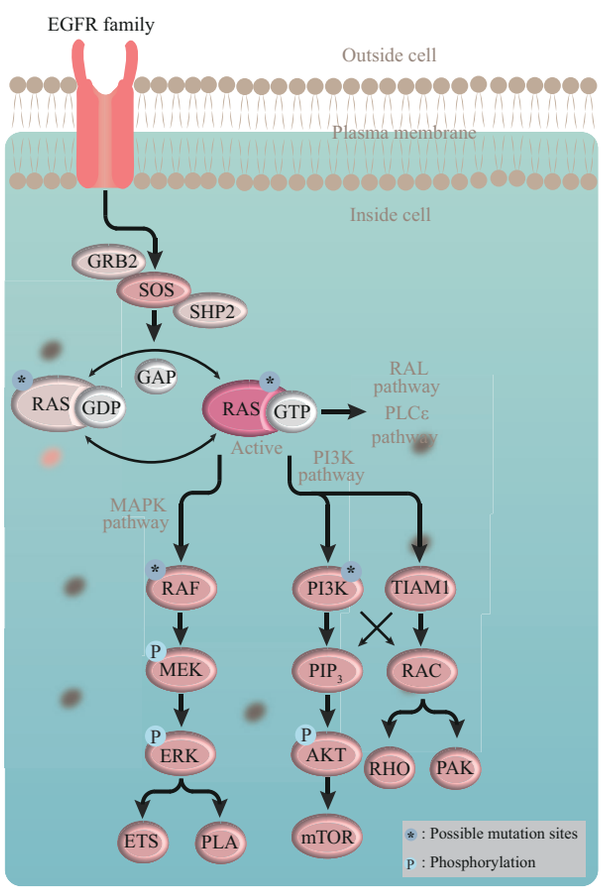

Fig. 1 The RAS−related signaling pathways in colorectal cancer

RAS proteins are monomeric GTP-binding proteins with weak GTPase activity related to the promotion of cell division and are important signaling

elements in the RAS-RAF-MAPK and PI3K-AKT-mTOR signaling pathways. Following the activation of receptor tyrosine kinases (RTKS, such as EGFR family members), the membrane valve cascade is launched. Then, RAS targets are activated to mediate the above two pathways and affect cell processes. EGFR is activated by EGF to form a domain that is phosphorylated, and then it binds to SHP2 on the aptamer GRB2 and causes SHP2 to undergo tyrosine phosphorylation.

When phosphorylated GRB2 binds to SOS, it activates SOS and promotes RAS protein activation[10]. A cascade of kinase activation (RAS-RAF(MAPKKK)-MEK1/2 (MAPKK)-MAPK (ERK) cascade) results from the activation of GTP-bound RAS proteins that bind to the c-RAF protein encoded by RAF. And it transmits the signal to the nucleus, which delivers the phosphorylation of a range of transcription factors to regulate gene expression. A batch of evidence does prove that overexpression of the RAS-RAF-MEK-ERK cascade is strongly linked to CRC, and the mutations that trigger this pathway activation mediate contact-dependent or dissolution-dependent interactions between the tumor-tumor microenvironment (TME) -immune system, turning out the establishment of immune escape mechanisms and tumor maintenance settings[11]. Thus, blockage of each kinase layer in this pathway inhibits cell growth and affects the tumor microenvironment and immune capabilities.

GTP-bound RAS proteins can also bind to the binding domain of p110 as catalytic subunit of PI3K, inducing the activation of downstream effectors of the PI3K-AKT-mTOR pathway and regulating transcription and translation processes during the cell cycle[12]. AKT activation not only maintains mTOR activity by phosphorylating and restricting TSC2, but it also regulates the MAPK signaling pathway by inhibiting RAF-1[13]. Furthermore, another target, RAC is regulated not just by RAS-regulated TIAM1, but also by PI3K, which is linked to the cytoskeleton and motility[14]. Of note, PIK3CA, the p110-α catalytic subunit of PI3K (with high probability in exons 9 and 20), is frequently mutated in CRC. The tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) is a direct antagonist of the pathway, and loss and mutation of PTEN expression have been shown to be highly related to poor prognosis in CRC[15].

In summary, the RAS-CRC medication development process has centered on three crucial components: inhibiting the activation of RAS as the core signaling pathway; inhibiting the activation of the RAS-RAF-MAPK pathway; and inhibiting the activation of the PI3K-AKT-mTOR pathway. Despite this, RAL and PLCε, as well as other associated targets, can regulate a wide range of cell life activities in the RAS protein downstream pathway[16]. At present, there are a variety of clinical compounds listed in the above three key links[17]. Although most of these compounds have shown anti-tumor efficacy, combination therapy, or synergistic therapy, remains the mainstream treatment for the majority of CRC patients.

2 Combinations of RAS−related agents for CRC in clinical trials

Due to the secondary mutation or gene amplification of the *RAS* gene, clinical drug resistance will occur. Targeted drugs combined with chemotherapy, the gold standard of treatment, have been shown to prolong the survival of patients with mCRC. Even in wild-type CRC patients, the effectiveness of drugs targeting RAS-related targets in combination with conventional therapies tends to be more attractive.

As first-line therapy for CRC, a series of chemotherapy regimens composed of calcium tetrahydrofolate and fluorouracil (5-FU) combined with different doses of oxaliplatin for the treatment of CRC is called the FOLFOX regimen. FOLFOX4, FOLFOX6, mFOLFOX6, FOLFOX7, FOLFOXIRI, and mFOLFOXIRI were all revealed in succession as a result of the ongoing development of clinical application after the De Gramont protocols, the predecessor of FOLFOX[18]. The American Society of Clinical Oncology (ASCO) has also highlighted the importance of chemotherapy regimens and their combination with EGFR inhibitors or VGFR inhibitors in the recommended first-line drug treatment for different molecular subtypes of mCRC (Table 1). In recent years, restrainers targeting EGFR or VEGF, the upstream signal of RAS, have always been used in conjunction with FOLFOX therapy to treat patients with RAS_{wt} or RAS_{mut} CRC[19].

Table 1 Recommended first-line drug treatments for different molecular subtypes of mCRC in American Society of Clinical Oncology (ASCO)[20-21]

No.	Molecular subtype of mCRC	Recommended treatment	Type	Evidence quality	Strength of recommendation	Replacement therapy
1	Initially unresectable MSS or pMMR	Doublet backbone chemotherapy: folinic acid, FU, oxaliplatin (FOLFOX)/FU, irinotecan (FOLFIRI)	Evidence-based, benefits outweigh harms	Moderate	Strong	Capecitabine plus oxaliplatin/ adding bevacizumab
		Triplet backbone chemotherapy: folinic acid, FU, oxaliplatin, and irinotecan (FOLFOXIRI)	Evidence-based, benefits outweigh harms	Moderate	Weak	Adding bevacizumab
2	Microsatellite instability-high or deficient mismatch repair	Pembrolizumab	Evidence-based, benefits outweigh harms	Moderate	Strong	—
3	MSS or pMMR left-sided treatm ent-naïve RAS wild-type	Anti-EGFR therapy (*i.e.*, panitumumab and cetuximab) plus doublet chemotherapy	Evidence-based, benefits outweigh harms	Moderate	Strong	Adding bevacizumab instead of anti-EGFR
4	MSS or pMMR right-sided RAS wild-type	Anti-VEGF therapy plus doublet chemotherapy	Evidence-based, benefits outweigh harms	—	—	—
5	RAS wild-type±BRAF mutation, patients with good PS and without major comorbidities, and/or when tumor shrinkage is the goal	Triplet backbone chemotherapy	Evidence-based	—	Moderate	Adding bevacizumab
6	RAS mutation	Doublet backbone chemotherapy	Evidence-based	—	Strong	Adding bevacizumab
7	BRAF V600E mutation that had progressed after at least one previous line of therapy	Encorafenib plus cetuximab	Evidence-based, benefits outweigh harms	Moderate	Strong	—
8	Any RAS status and dMMR or MSI-H and patients not candidates for intensive chemotherapy	Immune checkpoint inhibitors[1)]	Evidence-based	—	Moderate	—

[1)]Immune checkpoint inhibitors typically refer to monotherapy with pembrolizumab or nivolumab or nivolumab plus ipilimumab. BRAF: v-raf murine sarcoma viral oncogene homolog B1; dMMR: deficient mismatch repair; EGFR: epidermal growth factor receptor; FU: fluorouracil; mCRC: metastatic colorectal cancer; MSI: microsatellite instability; MSI-H: MSI-high; MSS: microsatellite stable; pMMR: proficient mismatch repair; PS: performance status; VEGF: vascular endothelial growth factor.

2.1 Combination strategies for wild-type CRC

2.1.1 Synergistic effect of cetuximab plus chemotherapy

Although the efficacy of an EGFR monoclonal antibody alone is significant in the treatment of RAS wild-type patients, the prognosis of combination therapy may be better. In a phase II study of cetuximab plus FOLFOX6, median progression-free survival (PFS) and overall survival (OS) were substantially higher in RAS_{wt}/$BRAF_{wt}$ mCRC patients with the left primary tumor after biweekly dosing[20]. And cetuximab combined with chemotherapy for left-sided malignancies led to a median overall survival of 34.3 months when used alone and 48.5 months when used in conjunction with neoadjuvant therapy, according to real-world research on Asian mCRC patients[21]. Another phase III study also suggested that the strategy of anti-EGFR with fluoropyrimidine (FP) was a better choice than anti-EGFR monotherapy[22].

Patients with technically unresectable RAS_{wt}/$BRAF_{wt}$ CRC with localized liver metastases were given mFOLFOXIRI plus cetuximab conversion

therapy, and the study showed surprising superiority: the rate of no evidence of disease was 70.1% in the cetuximab plus mFOLFOXIRI group and 41.2% in the modified-FOLFOXIRI group[23]. And the objective response rate (ORR), OS, and PFS were better with the synergistic regimen than with mFOLFOXIRI. First-line treatment with oxaliplatin and capecitabine (CapeOX) plus cetuximab also could achieve adequate ORR in RAS/BRAF/PIK3CA wild-type mCRC patients[24]. These results suggested that this combination therapy with the addition of cetuximab improves multiple indicators of mCRC, including ORR, PFS, OS, and patient-reported outcome (PRO), which can be used as a conversion strategy for patients with initially technically unresectable CRC liver metastases and even provide a favorable context for conversion to resectability. In a clinical study, oxaliplatin/5-FU/capecitabine combined with cetuximab was more effective and was safer as initial therapy than as alternative therapy. Unfortunately, some patients may develop KRAS mutations after prolonged cetuximab treatment, suggesting that KRAS mutations are associated with tumor progression and are dependent on the duration or dose of cetuximab treatment[25].

2.1.2 Synergistic effect of EGFR inhibitors plus chemotherapy

Other EGFR monoclonal antibodies (EGFR-mAb) have also been added to the combination therapy. A phase I study showed that panitumumab plus FOLFOXIRI chemotherapy was well-tolerated and had moderate anti-tumor activity in patients with mCRC[26]. Subsequent research found that the mFOLFOXIRI regimen, when paired with cetuximab or panitumumab in the randomized MACBETH and VOLFI trials, exhibited high effectiveness and tolerability in the treatment of RAS_{wt}/$BRAF_{wt}$ patients, respectively[27].

Regardless of RAS and BRAF molecular status, FOLFOXIRI with bevacizumab is regarded as a standard upfront therapy choice for clinically chosen patients with mCRC. Despite the lack of randomized comparisons, it is generally agreed that both FOLFOX-bevacizumab and FOLFOX-panitumumab are of value in the treatment of left-sided RAS_{wt}/$BRAF_{wt}$ mCRC, and no significant difference in efficacy was observed[28-29]. Numerically better survival outcomes were observed with FOLFOXIRI-bevacizumab than with FOLFOX-panitumumab, whereas chemotherapy-related adverse events were more common[28]. Interestingly, a Turkish group found that panitumumab combined with mFOLFOX6 was more effective than bevacizumab combined with mFOLFOX6 in the first-line treatment of wild-type RAS mCRC patients by analyzing patient-level PFS, OS, treatment dose, duration, use of subsequent treatments, occurrence of adverse drug events (ADEs), as well as the incidence and probability of metastasis resection in PEAK phase II clinical trials[30]. However, there is unremitting debate regarding the ideal first-line therapeutic regimen for individuals with mCRC following an objective response. The populations of the molecularly chosen RAS_{wt}/$BRAF_{wt}$ are more concerned about this issue. Once disease control is achieved by combination therapy including EGFR-mAb, further exposure to cytotoxic agents and targeted therapies may only lead to increased toxicity. Therefore, there is still room for improvement in the combo regimen *via* more clinical studies[31].

The optimal sequence of targeted therapy for patients with RAS_{wt} left-sided mCRC is still controversial, and there are few studies on the effect of first-line targeted drugs on second-line targeted drugs. One study, combined with previous clinical data, suggested that first-line cetuximab might provide a favorable condition for subsequent improvement in efficacy with bevacizumab, thus representing the best targeted therapy sequence for patients with RAS wild-type left-sided mCRC[32]. In a retrospective analysis of 450 mCRC patients given mFOLFOXIRI, the addition of anti-EGFR agents was beneficial with respect to PFS but not to median overall survival (mOS) in patients with left-sided $KRAS_{wt}$ mCRC[33].

2.1.3 Synergistic effect of EGFR inhibitors plus others

The overexpression/amplification of MET, a member of the receptor tyrosine kinase family, is associated with resistance to EGFR therapy in patients with mCRC. Clinical activity of combination EGFR and MET inhibitors in the treatment of mCRC has been established. Cabozantinib with or without panitumumab was linked to tumor-triggered MET amplification and upregulation of the TGF-axis in RAS_{wt} mCRC in a nonrandomized, open-label phase

Ib/II study[34]. TGF-β and c-MET signaling inhibition may provide a novel therapeutic approach for MET-amplified mCRC. Besides, the combination of the MET-RTK inhibiting agents tivantinib and cetuximab showed modest activity in EGFR-resistant mCRC wild-type patients with high MET expression, achieving an objective response in nearly 10% of patients in a refractory setting, but unfortunately the primary endpoint was not met[35]. In the afore-mentioned kinds of mCRC, the combination of capmatinib and cetuximab has also been shown to have preliminary effectiveness and excellent tolerability; however, the statistically significant results need to be further investigated[36].

Recent studies have shown that the mutation status of many downstream effectors, such as RAS and BRAF, is important in cetuximab monotherapy or combination therapy. Cetuximab, when combined with SN38, may increase SN38 sensitivity to RAS_{wt} CRC cells by inhibiting HSP27 and blocking the JAK/STAT signaling pathway[37]. Combination therapy with cetuximab and irinotecan has a beneficial and synergistic effect in patients with RAS_{wt} mCRC. Furthermore, in a study evaluating the safety and efficacy of panitumumab in combination with trifluridine and tipiracil (FTD/TPI) in patients with RAS_{wt} mCRC refractory to standard therapy (in addition to anti-EGFR therapy), this regimen demonstrated good antitumor activity and a manageable safety profile, suggesting that it could be used as a switching option for previously treated mCRC patients[38].

Unfortunately, cetuximab plus pembrolizumab is ineffective in patients with RAS_{wt} mCRC, despite partial local immune efficacy[39]. Further development of combined immune-tumor therapies with enhanced efficacy and/or targeting other or alternative immune checkpoints warrants investigation.

2.2 Combination strategies for mutant CRC

However, most patients have disease progression after first-line therapy and are eligible for second-line therapy. It is well known that for RAS_{mut} mCRC, EGFR-mAb alone has no benefit and has a poor prognosis. Patients with mCRC with KRAS exon 2 mutations and other RAS_{mut} do not benefit from anti-EGFR therapy. Multigene analysis of around 1 000 samples revealed that KRAS, NRAS, BRAF, and PIK3CA mutations accounted for 56.2%, 4.2%, 9.6%, and 16.8%, respectively, with coexisting mutations found in more than 10% of the samples[40]. More detailed data from COSMIC mutation database also sheds light on this issue (Figure 2). The ORR and DCR in wild-type and mutant KRAS patients were 42% (25/60) *vs* 11% (3/27) (P<0.05) and 60% (36/60) *vs* 26% (7/27) (P<0.05) in treatment with cetuximab plus chemotherapy[41]. The mOS and PFS of wild-type KRAS patients were significantly higher than those of mutant KRAS patients (21 months *vs* 17 months, P=0.017; 10 months *vs* 6 months, P=0.6). Given the high frequency of $KRAS_{mut}$ in mCRC patients, identifing whether $KRAS_{mut}$ occurrence is necessary when using cetuximab and other EGFR-mAb.

In a prospective retrospective analysis, panitumumab combined with FOLFOX4 resulted in shorter PFS and OS in Ras_{mut} patients than in wild-type[42]. As for a few mutation types (*e.g.*, MAP2K1 mutations), combining EGFR inhibitors with MEK, BRAF, or ERK inhibitors may not provide any clinical benefit[43]. In this setting, anti-angiogenic and anti-EGFR agents and immune checkpoint inhibitors have been approved as second-line treatment options, as well as $KRAS_{mut}$/$BRAF_{mut}$ and microsatellite status, which represent the molecular drivers that guide treatment selection. Patients with $KRAS_{mut}$ and $NRAS_{mut}$ are not candidates for anti-EGFR therapy, and bevacizumab is the only anti-angiogenic agent that improves survival in the first-line setting of combination chemotherapy, regardless of RAS_{mut} status.

As a recombinant humanized monoclonal antibody targeting VEGF, bevacizumab has shown promising preliminary benefits when combined with chemotherapy drugs. Continuation of bevacizumab after disease progression or its replacement with other anti-angiogenic agents has been shown to improve survival, whereas EGFR-mAb have become only an option for RAS_{wt} CRC. Recent studies have shown that as the first-line chemotherapy for patients with mCRC, the OS of the S-1 regimen and irinotecan combined with bevacizumab was equivalent to that of mFOLFOX6/CapeOX combined with bevacizumab, and the PFS was not inferior to that of mFOLFOX6/CapeOX[44], regardless of tumor side or RAS status. A non-randomized, single-center phase II trial was conducted to prospectively evaluate the combination of mFOLFOXIRI in combination with bevacizumab as a conversion therapy for initially unresectable RAS/BRAF/PIK3CA mutation mCRC. Results showed that

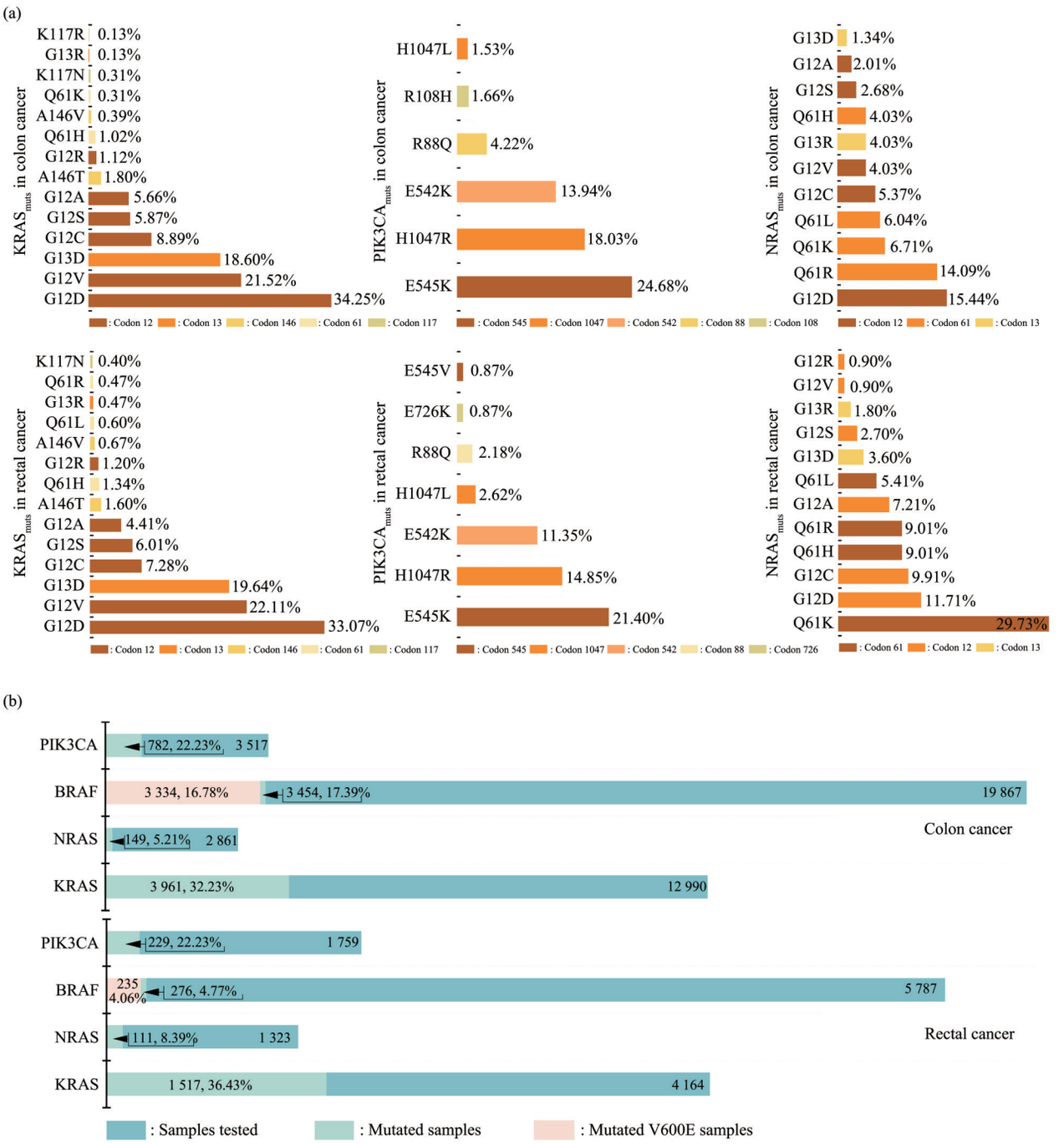

Fig. 2　Distribution of common RAS−associated mutations in colon and rectal cancer from COSMIC mutation database

(a) The proportion of each mutation site in the total mutation sample for KRAS, PIK3CA, and NRAS. (b) The proportion of KRAS, PIK3CA, and NRAS mutations for the two cancers and the proportion of mutated BRAF V600E to the total sample tested. COSMIC: public Catalog of Somatic Mutations in Cancer.

mFOLFOXIRI combined with bevacizumab increased the incidence of clinically evidence-free disease and showed a tendency to improve survival compared with mFOLFOXIRI alone. This may be a conversion treatment option for patients who cannot initially remove the RAS/BRAF/PIK3CA mutation in mCRC[45]. But in this context, the treatment benefit in RAS-mutant mCRC would differ from that in RAS_{wt} mCRC[46].

In addition, specific molecular subgroups, such

as $BRAF_{mut}$ and microsatellite instability-high (MSI-H) mCRC, represent aggressive malignancies that respond poorly to standard therapy and merit targeted therapy[47]. As an alternative therapy for $BRAF_{mut}$ mCRC, triple therapy with cetuximab, encorafenib (a BRAF inhibitor), and binimetinib (a MEK kinase inhibitor) improved OS by about 3 months compared with chemotherapy alone[48]. And the PFS of MSI-H mCRC patients was improved by 8 months with the addition of EGFR inhibitors to pembrolizumab, as compared with FOLFOX or FOLFIRI[48].

3 Combinations of RAS-related agents for CRC in pre-clinical study

Around 60% of mCRC have this mutation, which includes the *RAS/BRAF/PIK3CA* gene[49]. RAS, as well as its upstream and downstream related gene mutations, have brought many limitations to drug monotherapy. Therefore, many research groups have adopted new targeted combination therapy strategies in preclinical trials in order to solve the problem of drug resistance, and among them some strategies have produced positive outcomes (Figure 3).

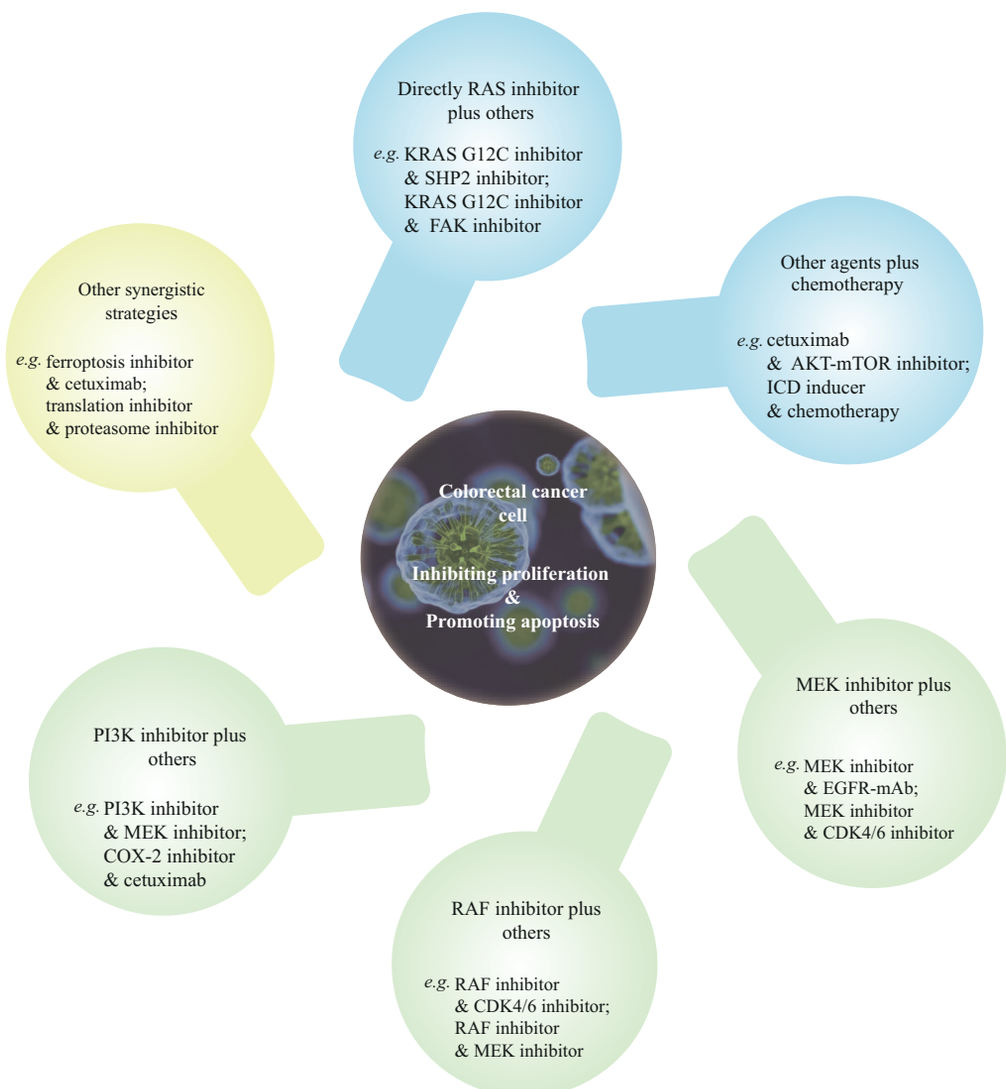

Fig. 3 Combination strategies of RAS-related agents for colorectal cancer in pre-clinical study

3.1 Synergistic drug delivery targeting RAS and its upstream

3.1.1 Synergistic effect of direct RAS inhibitors plus others

In patient-derived xenografts (PDX) of $KRAS_{mut}$ tumors, the KRAS G12C inhibitor MRTX849 has been shown to be more effective in combination with a SHP2 inhibitor than either alone[50], and such combination strategies have been investigated in clinical trials. Combining KRAS G12C inhibitors (AMG510 or MRTX849) with focal adhesion kinase (FAK) inhibitor IN0018 also demonstrated synergistic antitumor effects in multiple cancer cell lines (non-small-cell lung cancer, CRC, and pancreatic cancer) with KRAS G12C mutation, as well as in comparable cell derived xenograft and PDX models[51]. What's more, small molecule RAS inhibitors combined with siRNA, shRNA, and CRISPR technology have shown promising therapeutic application prospects in $KRAS_{mut}$ tumors[52-53].

Even so, it has been demonstrated that anti-tumor action was also observed with the simultaneous application of oligo-deoxyribonucleotides (ODNs) by multiple enzymes, as phosphorylation of ODNs facilitates their better entrance into cells. The combined use of one anti-telomerase ODN (Telp5) and two anti-KRAS ODNs (AS-KRAS and ISIS) at 20 μmol/L for 48 h reduced the survival rate of CRC cells by 99.67%, according to *in vitro* cell tests[54]. And studies have also proved that inRAS37, a pan-RAS-targeting IgG antibody, combined with YAP1 inhibitors, exhibited synergistic anti-tumor effects both *in vitro* and *in vivo*[55]. For individuals with malignancies that are drug-resistant, this synergistic therapy may provide hope for an alternate treatment.

3.1.2 Synergistic effect of RAS upstream inhibitors plus others

Besides these combinations of direct RAS inhibition, other biological agents or small compounds combined with chemotherapy medicines have shown noteworthy preclinical advantages. Previous studies have indicated that cetuximab, unlike other anti-EGFR antibodies, induces immunogenic cell death in combination with chemotherapy agents and leads to an increase in tumor infiltration of $CD8^+$ T and NK cells[56]. Coincidentally, the combination of dovitinib (a highly orally bioavailable III−V receptor tyrosine kinase inhibitor) and oxaliplatin increased *in vitro* cytotoxicity in colon cell lines and xenograft models relative to monotherapy, regardless of RAS-RAF status[57]. In a PDX model with a multidrug resistance phenotype, the combination also showed anti-tumor effects, as this strategy could inhibit MAPK and AKT pathways as well as induce apoptosis through caspase 9/caspase 3 activation. Another anti-IGF-IR monoclonal antibody, figitumumab, produced significant inhibitory effects on mouse CRC cells by inhibiting tumor cell survival while up-regulating chemotherapy (5-FU and gemcitabine)-induced apoptosis[58].

In syngeneic and genetically modified $KRAS_{mut}$ tumor models, combined treatment with a statin (an ICD inducer) and oxaliplatin greatly increased the immunogenicity of $KRAS_{mut}$ tumors and stimulated tumor-specific immunity[59]. Along with immune checkpoint inhibitors, this combination abolished PD-1 blockade resistance and increased survival in $KRAS_{mut}$ tumor mice. It is also usual to investigate novel therapeutic approaches using immunotherapy. For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) could boost the immune response to a vaccination containing a mutant RAS peptide to exert anti-tumor effects[60]. And cetuximab paired with the immune checkpoint inhibitor avelumab, two medications that trigger the activity of innate immune effectors, have a synergistic effect, offset the negative feedback of immunosuppression, boost the efficacy of each treatment, and cause apoptosis[61].

The pairing of cetuximab with the mTOR suppressant PP242 in BRAF wild-type CRC cells reduces tumor growth and proliferation *via* suppressing the phosphorylation of key molecules of MEK-ERK and MEK 4/7 (MKK) -c-Jun N-terminal kinase (JNK) signaling pathways downstream of EGFR[62]. In addition, treatment with 1 μmol/L cetuximab plus 1 μmol/L AZD5363 (an AKT inhibitor) or 1 μmol/L everolimus (an mTOR inhibitor) significantly inhibited RAS_{wt} and $PIK3CA_{mut}$ PDCS derived from CRC and downregulated AKT and ERK pathways in patient-derived cells (PDCs) line models derived from people with refractory colon cancer[63]. *In vitro*, cetuximab was shown to be insensitive to $KRAS_{mut}$ SW1463 cells; on the contrary, 4-acetyl-antroquinonol B (4-AAQB) successfully re-sensitizes CRC cells to cetuximab by diminishing colony formation, invasion, tumor-sphere formation,

and the tumorigenic KRAS signaling chain in CRC cells[64]. But in fact, this synergistic therapy had no impact on wild-type cells. More in-depth studies in mice using a xenograft model showed that tumor loads were dramatically reduced due to a combination of 4-AAQB and cetuximab. For this, miR-193a-3p and hsa-miR-324-5p production was enhanced in plasma, whereas tumor markers (EGFR) and p-MEK, p-ERK, and c-RAF/p-c-RAF signaling were repressed.

3.2 Synergistic drug delivery targeting RAS−RAF−MAPK and PI3K−AKT−mTOR pathways

3.2.1 Synergistic effect of MEK inhibitors plus others

Colorectal carcinogenesis is mostly attributed to the persistent activation of the RAF, MEK, and ERK pathways, as well as the PI3K and AKT pathways, by mutations in KRAS, a prominent subtype of RAS; hence, effective targeted treatments are desperately required. In recent years, the application of patient-derived organoids (PDOs) culture technology has further improved the accuracy of composite prediction tests, which can show the high incidence of $KRAS_{mut}$ and more closely capture the genetic diversity of tumors in a real environment[65]. Both normal and tumorigenic organoids showed a clear association between the presence of mutant RAS and resistance to targeted treatment. Most inhibitors, such as MEK inhibitors and RAF inhibitors, are largely ineffective when used alone, probably because of both primary and secondary resistance mechanisms[66]. Even after double blocking of the EGFR-MEK-ERK pathway, RAS_{mut} PDOs exhibit temporary cell cycle arrest but no cell death. Growth cycle arrest with pan-HER/MEK combo treatment was also demonstrated by the *in vivo* pharmacological response.

$KRAS_{mut}$-positive PDOs were shown to be robust to MEK antagonists, but BRAF class 3-positive PDOs responded fairly well to these drugs[67]. In a trial[68], researchers examined the cumulative influence of the anti-MEK antibody trametinib and the anti-EGFR antibody cetuximab with different RAS statuses *in vivo*. Trametinib alone or in conjunction with cetuximab promoted cell death and decreased ERK activation, despite the fact that cetuximab had no impact on the survival of RAS cells or ERK phosphorylation. In a more pathological cell delivery context, administration of trametinib and cetuximab to biopsy specimens from patients with KRAS colon cancer also showed less ERK phosphorylation. Individuals with KRAS exon 2 mutations experienced substantially longer PFS compared to previously treated patients in clinical trials, including anti-EGFR and anti-MEK. One caveat, however, is that targeting downstream effector molecules of RAS, including MEK and ERK, has had minimal therapeutic success in patients with $KRAS_{mut}$ cancers. The reduced sensitivity of $KRAS_{mut}$ cells to particular MEK antagonists (MEKi) is coupled with the feedback phosphorylation of MEK by C-RAF and reactivation of MAPK signaling[67].

In an intriguing combination clinical study, the MEK antagonist binimetinib plus the CDK4/6 inhibitor palbociclib, which hindered cell cycle progression, resulted in 60% tumor shrinkage in 18 PDX models, meeting the primary endpoint of the combined clinical trial[9]. The prolonged duration of response occurred mainly in the transplantation model with wild-type TP53. In addition, long-term continuous therapy in the PDX model is prone to issues with tyrosine kinase receptor feedback activation and acquired resistance, which can be reversed by SHP2 inhibitors[69]. Notably, the small-molecule SOS1 inhibitor BI-3406 binds to the catalytic domain of SOS1, reduces GTP-loaded RAS formation, and limits multiple KRAS-driven cancer cell proliferation. This mechanism of action could attenuate MEK inhibitor-induced feedback reactivation, thereby enhancing the susceptibility of KRAS-dependent cancers to MEK suppression[70]. An innovative and effective therapeutic idea for the administration of KRAS-driven malignancies is the simultaneous restriction of SOS1 and MEK. In future research, coupling KRAS agents, primarily SOS1 and SHP2 blockers, with other signal transduction inhibitors, such as MEK inhibitors or anti-EGFR antibodies, may yield therapeutic benefits beyond those obtained by each agent alone in the treatment of RAS-derived mCRC[71].

Additionally, dual inhibition of MEK1/2 and autophagy (trametinib with chloroquine) slowed down the progression of mCRC cell lines *in vitro* and assisted in the healing of tumors implanted in nude mice[72]. Inhibiting KRAS-RAF-MEK-ERK signaling raises the LKB1-AMPK-ULK1 signaling axis, a crucial regulator of autophagy, which is the mechanistic basis for how autophagy is triggered by preventing these pathways.

3.2.2 Synergistic effect of RAF inhibitors plus others

Direct inhibition of RAF by monotherapy rapidly leads to feedback activation of MEK, which inhibits the predicted regulation of apoptosis in tumor cells[73]. Tumors containing RAS or RAF gene variants may answer well to medications that disrupt combined RAF and d-cyclin-dependent kinases, as shown in recent research. Abemaciclib (a selective CDK4/6 blocker) and LY3009120 (a pan-Raf and RAF dimer inhibitor) synergistically suppressed the tumor cell population *in vitro* and resulted in tumor growth attenuation in xenograft models bearing KRAS, NRAS, or BRAF mutations[74]. Combination therapy with both drugs was well tolerated. Further *in vitro* testing of 328 tumor cell lines showed that cells with KRAS, NRAS, BRAF, or cyclin D activation were more sensitive to this combination, but cells with PTEN, PIK3CA, PIK3R1, or retinoblastoma (Rb) mutations were more resistant to it. Mechanistic investigations revealed that the combination of LY3009120 and abemaciclib completely suppressed Rb phosphorylation and cyclin D1, leading to a more dramatic G0/G1 arrest of the cell cycle.

The RAF dimer inhibitors lifirafenib (bgb283) and compound C have been demonstrated to have substantial proliferation synergy with MEKi, such as mirdametinib (PD-0325901) and selumetinib, in eliminating KRAS mCRC cell lines[75]. Analysis reveals that RAF dimer inhibition affects RAF-dependent MEK reactivation and leads to sustained inhibition of MAPK signaling in $KRAS_{mut}$ cells. This synergy was also observed in several $KRAS_{mut}$ mouse xenograft models. Pharmacodynamic analyses also support a role for synergistic phosphorylated ERK blockade in enhancing antitumor activity observed in the KRAS mutant model. However, this synergistic effect was not observed when vemurafenib (V600E), a selective B-RAF inhibitor, was used instead. Therefore, the combination of RAF dimers and MEKi for vertical inhibition strategies targeting $KRAS_{mut}$ cancers is also under clinical phase Ib/II investigation.

Furthermore, mechanisms of innate or acquired resistance to RAF inhibition generated by Ras mutant cancer cells are frequently mediated by ERK feedback activation. Therefore, an effective strategy is to combine ERK inhibitors, such as LY3214996 or MK-8353, to effectively overcome the ERK activation caused by using BRAF inhibitors and MEK inhibitors in $KRAS_{mut}$ tumors, and a synergistic benefit has been demonstrated[76].

3.2.3 Synergistic effect of PI3K inhibitors plus others

The PI3K signaling pathway frequently works independently of the RAS-MEK-ERK signaling system to encourage the development of tumor cells. Studies have shown that silencing KRAS in the treatment of $KRAS_{mut}$ colon cancer can effectively inhibit the activation of ERK, but does not affect the activation of PI3K-AKT signaling pathway[77]. In fact, co-inhibition of MEK and PI3K did have a notable synergistic impact on KRAS-independent tumors in this situation[78]. According to prior research, RAF-MEK-ERK and PI3K-AKT-mTOR co-inhibition may frequently result in superior clinical and therapeutic outcomes in the treatment of $KRAS_{mut}$ cancers.

In addition, *in vitro* evidence for PIK3CA-induced cyclooxygenase 2 (COX-2) expression was found, and a COX-2 inhibitor was useful in preventing colorectal adenoma and cancer. The precise mechanism of this anticancer activity is not known; however, it is assumed to include an interaction with the arachidonic acid metabolic pathway to decrease COX-2 synthesis[79]. Celecoxib, a specific COX-2 inhibitor, was added, which increased inhibitory effect of cetuximab on CRC cell proliferation and the induction of apoptosis, reduced tumor volume more effectively than monotherapy, increased the inhibitory effect of the EGFR signaling pathway, and decreased the interaction between β-catenin and FOXM1[80].

3.3 Other synergistic strategies targeting RAS−related pathways

For the treatment of mCRC, the possibility of combining ferroptosis inhibitors with conventional medications has also been investigated. It was shown that cetuximab increased the cytotoxic effects of RSL3 on KRAS-mutant CRC cells as well as the inducer of RSL3-induced ferroptosis *via* blocking the Nrf2/HO-1 axis through activation of p38 MAPK. Interestingly, RAS signaling pathways, MAPK signaling pathways, PI3K-Akt signaling pathways, and JAK-STAT signaling pathways were considerably enriched in CRC patients with greater ferroptosis related genes (FRGs) risk scores, and these patients were more susceptible to RSL3[81]. Additionally, combining vitamin C (VitC) with cetuximab inhibited the growth of CRC organoids and markedly

postponed the development of acquired resistance in xenografts made from CRC patients[82]. Mechanistically, this combination could effectively limit the emergence of anti-EGFR antibody acquired resistance by triggering a synthetic lethal metabolic cell death program through ATP depletion and oxidative stress. The above conclusion was also found when natural products elemene and cetuximab were combined[83].

Furthermore, by triggering estrogen receptor (ER) stress and extrinsic apoptotic pathways, a combination of translation inhibitors and proteasome inhibitors has been proven to cure advanced colon cancer. When Episilvestrol and Bortezomib were co-targeted, RAS/RAF-mutant colon cancer cells in mice died quickly and underwent apoptosis but pro-survival factors such p-AKT, p-4EBP1, mcl1 and eiF4E target proto-oncogenes, as well as Bcl-xL, were maintained or even increased[84].

4 Drug resistance in combination with RAS−related agents

The incidence of CRC-related death has a relationship to the development of secondary resistance after an initial response to treatment, particularly targeted therapies such as EGFR-mAb used in second- and third-line settings in patients with KRAS/NRAS wild-type mCRC. The combination of cytotoxic chemotherapy and targeted therapy is still the backbone of treatment for patients with metastatic cancer, although developed resistance will inevitably shorten the length of the response[85].

Patients with CRC who develop resistance to EGFR inhibitors (EGFRi) often have new mutations in KRAS, NRAS, or EGFR. Acquired mutations were more widespread among those whose cancers reacted to EGFRi alone (46%) than among those whose tumors responded to combination cytotoxic chemotherapy (9%)[86]. Single-agent EGFRi treatment resulted in a larger prevalence of acquired MAPK mutations than combination therapy, although transcriptome mechanisms of resistance were more prominent when aggressive cytotoxic chemotherapy was present[87]. Animal models have exhibited comparable outcomes to this clinical phenomenon, those who have acquired resistance to cetuximab or chemotherapy may often develop cross-resistance to alternative agents, with a transcriptome profile compatible with epithelial-to-mesenchymal transition[88]. Alternatively, susceptibility to cytotoxic treatment is unaffected by the kinds of acquired resistance that include changes in the MAPK pathway.

A mechanistic justification for investigating the joint use of anti-EGFR and vitamin C treatment to specifically target the critical resource itself (EGF) and the important resource acquisition player (glucose transporter) in CRC can be described as the differential MAPK signaling activity and ascorbate and calcium metabolism levels in the front and center of the tumor[89]. More recently, xenografts created from CRC patients have verified this theory. Combining cetuximab with vitamin C in a CRC RAS/BRAF wild-type model suppressed and postponed the appearance of secondary resistance to EGFR blockers.

MEK inhibition has gained widespread acceptance as an approach for RAS-mutant CRC. Yet, this novel agent’s therapeutic value is diminished by patients’ adaptive tolerance to MEK inhibitors. Restraint of SRC, a frequent signal transduction node, may be a mechanism for reducing MEK resistance[90]. Thus, SRC depressants may be useful in avoiding the development of resistance to MEK inhibitors. Gene expression signature scores for RAS pathway activation and MEK inhibitor tolerance may also be helpful indicators for predicting the likelihood of a favorable response to the novel trametinib and dasatinib combo therapy for CRC[91].

Anti-EGFR and MAPK suppressants work together to halt the progress of EGFR-mAb tolerance in KRAS wild-type CRC, while reversing initial resistance to anti-EGFR in KRAS-mutated CRC cell lines. However, rapid drug resistance in KRAS-mutated CRC is a limitation of combination therapy. Interestingly, activation of the PI3K-AKT pathway was shown to be an escape route in cell lines with acquired drug resistance following the simultaneous suppression of EGFR and MEK. Pharmacological blockage of the AKT pathway alone is insufficient to restore the resistant phenotype since it is related to the activation of numerous RTKS, including HER2, HER3, and IGF1R. An autocrine loop and heterodimers of several receptors function to activate the PI3K pathway. In KRAS mutant CRC cell lines, PI3K activation is a key mechanism underlying acquired resistance to the combined use of anti-EGFR and MEK depressants. PI3K activation is co-mediated by the activation of multiple RTKs such as HER2,

HER3, and IGF1R[92].

What's more, it was found that immunomodulatory oligonucleotide (IMO), when combined with cetuximab, it dramatically restored the sensitivity of KRAS mutational cancer cells to cetuximab, considerably reduced cell survival, and completely suppressed mitogen-activated protein kinase phosphorylation. IMO modulates the functional connection between TLR9 and EGFR via interfering with EGFR-dependent signals. Together, IMO and cetuximab showed strong and durable synergistic anticancer efficacy in LS174T CRC *in vivo*. Further evidence from homologous recombination of KRAS mutant CRC cell types showed that IMO could replace cetuximab susceptibility. IMO, together with cetuximab as a treatment strategy for KRAS wild-type and KRAS mutated cetuximab-resistant CRC, this can be a focus of consideration in subsequent studies[93].

5 Pharmacovigilance studies in combination with RAS−related agents

Targeted therapy combined with chemotherapy significantly increased the risk of serious adverse events (SAEs), and comprehensive pharmacovigilance studies still need to be strengthened in the treatment of RAS-derived CRC involving chemotherapy. In the Phase III GONO trial, FOLFOXIRI regimens also resulted in markedly elevated grade 2 or 3 peripheral neurotoxicity (0% *vs* 19%, $P<0.001$) and grade 3 or 4 neutropenia (28% *vs* 50%, $P<0.001$) when compared to FOLFIRI regimens, despite having a high response rate with 5-FU, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) triple chemotherapy[94]. The prevalence of hematological, gastrointestinal, and nervous system SAEs did not differ significantly ($P>0.05$) between the single dose of targeted medicines and placebo in one trial[95]. Bevacizumab-based chemotherapy, on the other hand, increased the risk of severe hypertension ($P<0.05$), and panitumumab-based chemotherapy raised the risk of severe thromboembolism (RR 3.65; 95%CI 1.30−10.26). Compared to cetuximab-based chemotherapy, panitumumab-based chemotherapy had a relatively greater incidence of skin damage (RR 15.22; 95% CI 7.17−32.35), mucositis (RR 3.18; 95% CI 1.52−6.65), hypomagnesemia (RR 20.10; 95% CI 5.92−68.21) and dehydration (RR 2.81; 95% CI 1.03−7.67).

Patients with KRAS wild-type mCRC in Turkey had a worse outcome when treated with panitumumab-containing combinations compared to those treated with bevacizumab-containing or cetuximab-containing combinations, according to a retrospective assessment of first-line therapy regimens. Patients who were given FOLFOX-bevacizumab were more likely to suffer from neuropathy and neutropenia as side effects, while those who got FOLFIRI-bevacizumab experienced neutropenia and perforation. Rash and pustular infections developed in cases who received FOLFIR-cetuximab; Diarrhea occurred in those receiving the FOLFIRI-panitumumab combination[96]. Further debate is required, however, about how to quantify safety and clinical benefit. Mitomo *et al.*[97] also suggested that the adverse effects of panitumumab combined with chemotherapy may be manageable if the strategy results in tumor shrinkage and hopefully resection of metastatic lesions.

6 Conclusion

Due to the complexities of intracellular signaling network control, single suppression of RAS and its upstream and downstream signaling pathways frequently results in compensatory activation of other signaling pathways and tumor drug resistance, approaching a bottleneck in treatment[98]. In recent years, RAS-related signaling pathway inhibitors have been utilized in conjunction with other medications with promising outcomes in clinical trials and preclinical investigations. Among them, EGFR inhibitors, VEGF inhibitors, RAS direct inhibitors, MEK inhibitors, and RAF inhibitors have the most prominent efficacy in combination with other anti-tumor drugs. On the one hand, clinical trials combining cetuximab, an EGFR-mAb, and bevacizumab, an VEGF-mAb, are actively being conducted. On the other hand, the dominant approach to address the medication resistance issue in the future is still the combination of targeted RAS downstream pathway proteins in preclinical studies. Even so, it is important to note that the need for adopting personalized medication strategies for mCRC patients with various molecular subtypes is becoming more evident. This will necessitate a substantial body of preliminary experimental data, and the current research in this area is still somewhat insufficient.

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RAS相关通路介导的结直肠癌药物联合治疗的应用*

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摘要　RAS相关信号通路在结直肠癌的发生、发展中起着重要作用，与该类肿瘤细胞的增殖、转移、凋亡密切相关。目前，包括靶向药物、化疗药物的单药治疗对结直肠癌的临床获益并不理想。近年来，在临床试验和临床前研究中RAS相关信号通路的抑制剂与其他药物的联合应用取得了良好效果，其中EGFR抑制剂、VEGF抑制剂、RAS直接抑制剂、MEK抑制剂和RAF抑制剂的表现尤为突出。本文就RAS相关信号通路与结直肠癌的作用关系、临床试验和临床前研究中的联合用药策略以及组合用药的耐药机制研究进行系统性综述，以期为未来临床多药治疗策略奠定基础。

关键词　RAS，结直肠癌，信号通路，表皮生长因子受体抑制剂，药物联合应用，耐药性
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