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**Strategies to overcome resistance to epidermal growth factor receptor monoclonal antibody therapy in metastatic colorectal cancer**

Woo-Jeong Jeong, Pu-Hyeon Cha, Kang-Yell Choi

**Woo-Jeong Jeong, Pu-Hyeon Cha, Kang-Yell Choi,** Translational Research Center for Protein Function Control, Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, South Korea

**Author contributions:** Jeong WJ and Cha PH wrote the manuscript draft and edited the final version of the article; Choi KY revised it critically for important intellectual content and approved the final version.

**Correspondence to: Kang-Yell Choi, Professor,** Translational Research Center for Protein Function Control, Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University,134 Shinchon-Dong, Seodemun-Gu, Seoul 120-749, South Korea. [kychoi@yonsei.ac.kr](mailto:kychoi@yonsei.ac.kr)

**Telephone:** +82-2-21236592 **Fax**: +82- 2-3627265

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**Abstract**

Administration of monoclonal antibodies (mAbs) against epidermal growth factor receptor (EGFR) such as cetuximab and panitumumab in combination with conventional chemotherapy substantially prolongs survival of patients with metastatic colorectal cancer (mCRC). However, the efficacy of these mAbs is limited due to genetic variation among patients, in particular *K-ras* mutations*.* The discovery of *K-ras* mutation as a predictor of non-responsiveness to EGFR mAb therapy has caused a major change in the treatment of mCRC. Drugs that inhibit transformation caused by oncogenic alterations of *Ras* and its downstream components such as BRAF, MEK, and AKTseem to be promising cancer therapeutics as single agents or when given with EGFR inhibitors. Although multiple therapeutic strategies to overcome EGFR mAb-resistance are under investigation, our understanding of their mode of action is limited. Rational drug development based on stringent preclinical data, biomarker validation, and proper selection of patients is of paramount importance in the treatment of mCRC. In this review, we will discuss diverse approaches to overcome the problem of resistance to existing anti-EGFR therapies and potential future directions for cancer therapies related to the mutational status of genes associated with EGFR-Ras-ERK and PI3K signaling.

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**Key words:** Colorectal cancer; Epidermal growth factor receptor; Resistance; *K-ras* mutation*;* combinational therapy

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**INTRODUCTION**

**Colorectal cancer (CRC) is the third most frequently diagnosed type of cancer and the leading cause of cancer-related deaths worldwide**[1,2]. **CRC is highly treatable when diagnosed and surgically removed at an early stage; however, 5-year survival is less than 10% in patients with unresectable metastasis[3,4]. Approximately 40%-50% of CRC patients develop metastatic cancer and 80%-90% of these have unresectable metastases[5]. Chemotherapy is usually suggested for the treatment of metastatic CRC (mCRC), because surgery is limited to patients who have no metastasis outside of the liver or those who would have an appropriate amount of liver left after the surgery[4]. Conventional chemotherapy such as** 5-fluorouracil (5-FU)/leucovorin (LV), irinotecan, or oxaliplatin is still mainly used as treatment for patients with mCRC[6]. Moreover, combinational therapy of oxaliplatin or irinotecan with 5-FU/LV has considerably improved the therapeutic outcome of this group of patients[7-10]. However, these chemotherapeutic agents have various adverse effects such as hair loss, nausea, and vomiting[11] because they interfere with the division or reproduction of rapidly growing normal cells such as bone marrow cells in addition to their desired effect on cancer cells.

The recent development of targeted or biological therapeutics represents a substantial advance in treatment for mCRC. Although the efficacy of these targeted therapeutics is restricted to certain individuals because the drugs work on specific target proteins, these approaches have critically improved the survival of patients with metastases. When used appropriately to treat patients according to their molecular profiles, targeted therapeutics significantly prolongs overall survival and disease-free survival. Moreover, these treatments showed fewer adverse effects such as hair loss and nausea than conventional chemotherapy. Most of the targeted therapeutic agents currently in development or in clinical usage are molecules with high affinity for growth factor receptors, such as epidermal growth factor receptor (EGFR)[4].

The recent introduction of monoclonal antibody (mAb) drugs targeting EGFR such as cetuximab (Erbitux; ImClone, Branchburg, United States) and panitumumab (ABX-EGF; Amgen, Thousand Oaks, United States), into combination chemotherapy regimens with currently used drugs for the treatment of mCRC patients has been shown to be effective and has widened treatment options. However, the efficacy of these two mAbs is limited by the unresponsiveness of patients harboring a *K-ras* mutation[12]. Here, we review the mechanisms underlying resistance to ERFR mAb therapies due to *K-ras* mutations and discuss the current status of drug development strategies to overcome the problem of resistance in the treatment of patients with mCRC.

**MONOCLONAL ANTIBODIES TARGETING EGFR FOR THE TREATMENT OF CRC**

***EGFR***

**The EGFR is a receptor tyrosine kinase (RTK) belonging to the ErbB family of cell membrane receptors. Binding of ligands, such as EGF or transforming growth factor alpha (TGFα) to EGFR induces dimerization and activation of the receptors. This RTK is auto-phosphorylated and induces activation of multiple downstream signaling pathways including** extracellular-signal-regulated kinase (ERK) **and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways (Figure 1). These two pathways are involved in the regulation of various cell physiological cellular processes such as proliferation, migration, apoptosis, and angiogenesis[13] (Figure 1). Therefore, dysregulation of EGFR signaling can induce malignant transformation and tumor progression through activation of downstream signaling.**

**EGFR is frequently overexpressed or mutated, and such changes are associated with tumor progression and poor prognosis in many types of cancers including head and neck cancers[14]. EGFR has also been shown to be highly overexpressed in 25%-82% of cases of CRC[15]. Although the clinical significance of EGFR overexpression or gene copy number in CRC is controversial, recent studies indicate that these genetic characteristics are associated with prognosis or survival of CRC patients[16-18]. Consequently, EGFR has attracted great attention in the field of anti-cancer drug development because of its presumed role in tumor growth and progression. Cetuximab and panitumumab, two monoclonal antibodies against EGFR, have recently been used in the treatment of mCRC patients and have shown effective clinical benefits in 10%-20% of patients[19-22].**

**CETUXIMAB AND PANITUMUMAB**

Cetuximab and panitumumab inhibit EGFR downstream signaling pathways, such as Ras/ERK and PI3K/Akt pathways. The safety and efficacy of these two mAbs has been studied in patients with EGFR-overexpressing CRC, and both agents have shown reliable efficacy in these patients**[19-22]**. In a randomized controlled trial including 329 patients who received either cetuximab (400 mg/m2 initial dose followed by 250 mg/m2) and/or the topoisomerase I inhibitor irinotecan[23], patients that receive both drugs had an objective response rate of 22.9% compared with 10.8% for those receiving only cetuximab. Cetuximab is used in combination with irinotecan to treat mCRC patients who are refractory to irinotecan-based chemotherapy[24]. It is also used as a single agent for mCRC patients with intolerance to the irinotecan-based chemotherapy[24]. The efficacy of panitumumab was studied in 463 patients who received panitumumab (6 mg/kg) with the best supportive care (BSC) or only BSC[25]. Panitumumab significantly prolonged progression-free survival (PFS) of patients treated with panitumumab and BSC compared with patients who received only BSC[25]; the mean PFS in patients receiving both panitumumab and BSC was 96 d whereas that of patients receiving BSC alone was 60 d[25]. Panitumumab is approved by the United States Food and Drug Administration (FDA) for the treatment of patients with EGFR expressing mCRC after following fluoropyrimidine-, oxaliplatin- and irinotecan-containing chemotherapy[26]. This agent is also approved by the FDA as a single agent for the treatment of mCRCs[26].

Although cetuximab and panitumumab have been shown efficacy in patients with EGFR-expressing mCRC, their benefit is restricted to only a small proportion (8%-23%) of patients because mCRC harboring a *K-ras* mutation is resistant to these mAbs. Therefore, the FDA suggested that the *K-ras* gene mutational status of mCRC patients should be evaluated prior to administration of EGFR mAbs to avoid wasting time and money[27].

**MECHANISMS OF RESISTANCE TO EGFR-TARGETED MABS**

Despite evidence of the efficacy of cetuximab in the treatment of mCRC patients, the low response rate was the proof of concept for resistance to treatment with anti-EGFR mAbs. There is a large body of evidence supporting the existence of negative predictors that identify patients who should not be treated with anti-EGFR mAbs. The identification of additional genetic determinants of primary resistance to EGFR-targeted therapies in CRCs is important to allow prospective identification of patients who should not be treated with cetuximab, thus avoiding their exposure to ineffective and expensive therapy. Recent work has therefore focused on the analysis of oncogenic mutations in genes encoding key downstream effectors of the EGFR signaling pathway[28,29].

***K-Ras***

*K-ras*, a member of the rat sarcoma virus (ras) gene family of oncogenes (which includes *K-ras*, *H-ras*, and *N-ras*), encodes the guanosine diphosphate (GDP)- and guanosine triphosphate (GTP)-binding protein Ras that acts as a self-inactivating intracellular signal transducer[30]. K-Ras act as an important, but not exclusive effector of EGFR[12,31], signaling mainly but not exclusively through BRAF and the ERK axis. K-Ras can also activate PI3K through direct interaction with its catalytic subunit[32]. When K-Ras is mutated, PI3K results in constitutive activation of its downstream signaling pathway that causes the cells to become independent of EGFR signaling activation. Somatic mutations of *K-ras* occur in 30%-40% of CRCs and predominantly in codon 12 (approximately 70%-80%) and codon 13 (approximately 15%-20%) of exon 2 (Table 1). *K-ras* mutations have emerged as the major negative predictor of efficacy in patients receiving cetuximab. Strong evidence that *K-ras* mutations are associated with the lack of response to cetuximab in chemorefractory mCRC patients led the FDA and the European Medicines Agency (EMEA) to restrict the use of cetuximab as monotherapy or in combination with chemotherapy, to patients with *K-ras* wild type (WT) tumors[33]. Clearly, the *K-ras* biomarker identifies CRC patients likely to benefit from anti-EGFR therapy. However, because only 20%–40% of patients with *K-ras* WT will respond to cetuximab, either in monotherapy or in combination therapy, *K-ras* status alone does not accurately predict the subset of CRC patients that will respond to EGFR mAbs.

***N-Ras***

Due to the low frequency of *N-ras* mutations in mCRC (approximately 3%-5%; Table 1), mutational status of *N-ras* have not been considered as predictive biomarkers in the treatment of mCRC to be applied for the anti-EGFR mAb therapy.

Recently, in a randomized phase 3 study of panitumumab monotherapy[34] and other preliminary findings[35-40] suggest that *N-ras* mutations are associated with the resistance to cetuximab and panitumumab. In the *K-ras* WT patients, carriers of *B-raf* and *N-ras* mutations had a significantly lower response rate than those harboring wild-type *B-raf* and *N-ras*[39]. Therefore, checking the mutational status of all *ras* isotype can provide additional predictive information for the prescription of EGFR mAb therapy in mCRC[40].

***B-Raf***

BRAF is a cytoplasmic serine/threonine kinase that directly interacts with Ras[41]. Known *B-Raf* mutations are mainly located in the kinase domain, with a single substitution of glutamic acid for valine at codon 600 (V600E) accounting for 80% of all mutations although other, less frequent, activating mutations affect the same residue, including V600A, V600D, V600G, V600K, V600M, and V600R[42]. The V600E amino acid substitution is thought to be responsible for the oncogenic properties of BRAF through insertion of a negatively charged amino acid in the activation segment, thus mimicking phosphorylation of the kinase and causing it to be constitutively active[42]. BRAF V600E is the most common point mutation in mCRC, and is present in approximately 10%-15% of cases **(Table 1)**[43]. K-Ras and BRAF function in the same pathway downstream of EGFR and mutations in the genes encoding these proteins are mutually exclusive, therefore one could speculate that the presence of an active mutation in one of these two molecules is sufficient to drive constitutive activation of the pathway[43]. Because RAF is an important downstream effector of Ras, targeting RAF could be an effective strategy for the treatment of *K-ras* or *B-Raf* mutated tumors. Surprisingly, vemurafenib (PLX4032), a selective BRAF inhibitor that showed pronounced efficacy in *B-Raf*-mutant melanoma patients, had only modest clinical activity in a study evaluating 19 mCRC patients with the BRAF V600E mutation, suggesting that the biology of BRAF activation in patients with mCRC is more heterogeneous than that in melanoma[44]. In other studies, no response to cetuximab or panitumumab was observed in patients with *B-Raf*-mutant mCRC[39,45].

***PI3K/PTEN***

One of the major pathways activated by EGFR is the PI3K/Akt signaling pathway. This pathway can be deregulated by either inactivation of the phosphatase and tensin homologue (PTEN) or by activating mutations of the PI3K p110 catalytic subunit (PIK3CA). Activation of PIK3CA results in increased AKT/mTOR pathway signaling and increased cellular proliferation[46]. Class IA PI3Ks are heterodimers composed of regulatory (p85) and catalytic (p110) subunits. Mutations in the PIK3CA gene occur in approximately 15%-18% **(Table 1)** of CRCs[47,48]. More than 80% of PIK3CA mutations in CRCs occur in exon 9 (60%-65%) or exon 20 (20%-25%)[39]. The gain of function induced by mutation in exon-9 (helical-domain) is independent of binding to the p85 regulatory subunit but requires interaction with Ras-GTP. In contrast, mutations in exon–20 (kinase–domain) are active in the absence of Ras-GTP binding but are highly dependent on the interaction with p85[49]. *PIK3CA* mutations can be found together with *K-ras* or *B-Raf* mutations in the same tumor, and this makes it difficult to evaluate their individual role in defining sensitivity to anti-EGFR mAbs[39,50]. Patients with mutation in *K-ras* or *B-Raf* and WT *PIK3CA* do not respond to cetuximab whereas patients with WT *K-ras* and *B-Raf* but mutations in *PIK3CA* may have different sensitivity depending on the characteristics of the mutations they harbor: *PIK3CA* exon 20 mutations are associated with resistance to cetuximab whereas *PIK3CA* exon 9 variants have no significant effect on response[39]. PI3K-initiated signaling is inhibited by PTEN. Recent reports suggested that inactivation of PTEN is associated with resistance to EGFR targeting agents[51,52]. However, the role of PTEN loss in CRC is unclear and the lack of a standardized method for PTEN detection limits the possibility of using this marker in the clinical setting.

**COMBINATIONAL THERAPIES**

Because of the crosstalk between many of the RTK signaling pathways, we do not expect a single gene dependency for cancer phenotypes. Moreover, cancer cells that are treated with drugs that block a single molecular target are often able to activate alternative pathways as escape mechanisms to overcome the blockade. In addition, the effectiveness of drugs varies depending on the mutational status of the relevant gene. Therefore, appropriate selection of patients for treatment with anti-EGFR drugs is a major challenge in the management of mCRC. Collectively, the data available from clinical studies suggest that these drugs are active only in a subset of patients. The most promising approaches are rational combinations of targeted treatments that include inhibitors of downstream effectors of the EGFR pathway. At the present time, several drugs that inhibit activated BRAF, MEK, PI3K, Akt, and mTOR are available and evaluations of these drugs in clinical trials are actively ongoing (Table 2)[53-77]. However, a comprehensive understanding of the precise role of these potential drug targets in CRC and the oncogenic dependence of tumors on these components is still lacking[78].

***RAS inhibition***

The Ras pathway is central to many nodes of RTK signaling and because it is constitutively activated in many human cancers including CRC, it can bypass the EGFR-driven signaling cascade and reduce the clinical efficacy of EGFR inhibitors. Therefore, development of new therapeutic strategies for CRC with mutant *K-ras*, is critically needed. Several different strategies have been applied to target oncogenic Ras. One of the initial strategies used was inhibition of Ras farnesylation, a posttranslational modification that is required for localization of Ras to the plasma membrane. Preclinical studies of farnesyltransferase inhibitors (FTIs) in transgenic mouse models that overexpress oncogenic Ras showed potent antitumor activity[79]. However, FTIs have shown little, if any clinical activity in patients with solid tumors, probably because of alternative modification of Ras such as geranylgeranylation[80,81]. As another approach to targeting oncogenic Ras, synthetic lethal screening has been used to identify novel anticancer agents capable of selectively killing tumor cells harboring a specific mutation[82-84]. The idea of reducing Ras expression by antisense or RNA interference is promising, but successful application of this technology is currently limited by lack of efficient delivery, uptake, and gene silencing. Using high-throughput screening approaches with loss-of-function RNAi, several groups have identified proteins that, when lost, elicit a synthetic lethal response when combined with mutant Ras oncogenes but have no effect on cells with WT Ras[85-89]. Despite all these efforts, there are still no effective therapeutic agents or regimens available in the clinic for patients with tumors associated with *K-ras* mutation.

***RAF inhibitors***

RAF is an important effector that functions downstream of Ras in the ERK signaling pathway and therefore represents a potential target for the treatment of tumors with mutnat *K-ras*. Although the BRAF V600E inhibitor vemurafenib (PLX4032) shows pronounced activity in patients with *B-Raf* mutated melanoma[44], the clinical activity of vemurafenib in previously treated patients with *B-Raf*-mutated mCRC was more modest, with a response rate of only 5% (one partial response, no complete responses) among 20 patients[44]. Interestingly, resistance to therapy in *B-Raf*-mutated CRC appears to be caused by persistent activation of the EGFR signaling pathway. Recently, two research groups independently reported that blockade of BRAF causes rapid feedback activation of EGFR[90,91], which upon phosphorylation triggers sustained activation of ERK signaling and cell proliferation through activation of Ras and CRAF. *In vitro,* inhibition of EGFR activity by cetuximab restores sensitivity to vemurafenib. Clinical trials of a combination of vemurafenib and cetuximab in metastatic *B-Raf*-mutated CRC are currently underway (Table 2)[92]. Additionally, resistance to BRAF inhibition may also develop through activation of other signaling pathways. CRC demonstrates a higher level of PI3K/Akt signaling than melanoma, and *B-Raf* mutated colorectal cells display lower sensitivity *in vitro* to vemurafenib in the presence of concomitant PTEN or PI3K mutations[93]. Although these findings confirm mutant *B-Raf* as a therapeutic target in this disease, they also show that the biology of BRAF activation is clearly more heterogeneous in CRC than in other tumor types.

***MEK inhibitors***

Selective inhibitors of MEK kinases seem an attractive target for tumors that preferentially signal through the Ras-RAF-MEK-ERK pathway. Proof of concept was provided in a preclinical study with the MEK inhibitors AS703026 or AZD6244 (Table 2), which inhibited the growth of xenograft tumors formed by CRC cells with mutant *K-ras*[94]. We further investigated the effect of MEK inhibitors on cells with cetuximab resistance attributed to *K-ras* mutation using isogenic DLD-1 CRC cell lines (D-WT and D-MUT) that harbor WT or mutant *K-ras* alleles respectively, and found that the MEK inhibitors suppressed cetuximab-resistance of CRC cells that was attributed to *K-ras* mutation both *in vitro* and *in vivo*[94]. Recent studies showed that a compensatory or activating feedback loop between RAF-MEK-ERK and PI3K pathways counteracts the effect of MEK inhibition[95]. Moreover, dual inhibition with MEK and PI3K inhibitors resulted in marked inhibition of tumor cell growth. *In vitro* studies in *K-ras*-mutant CRC cell lines showed that the presence of activating mutations in PIK3CA or loss-of-function mutations in PTEN resulted in insensitivity to MEK inhibitor[96]. These studies also showed that mutational activation of PIK3CA is not functionally equivalent to PTEN loss. Therefore, the authors concluded that PI3K pathway activation is a major resistance mechanism that impairs the efficacy of MEK inhibitors in *K-ras* mutated cancers, and it is therefore important to test whether pan-PI3K inhibitors will act synergistically with MEK inhibitors in cancers with coexisting PTEN and *K-ras* mutations. Together with the previous study, this provides a strong rationale for combination treatment with PI3K and MEK inhibitors. In conclusion, although MEK inhibition is theoretically an interesting approach to targeting *K-ras* activated tumors, it is very likely that MEK inhibitors will only be efficient in a subgroup of *K-ras* mutant CRCs. As we discuss in this manuscript, combination with other targeted agents is probably a more efficient approach.

***PI3K/Akt/mTOR pathway***

Inhibition of PI3K could be a feasible approach to the treatment of CRC that is resistant to EGFR monoclonal antibodies because of abnormal PTEN/PI3K status. Moreover, because the oncogene *K-ras* can activate the PI3K-Akt-mTOR pathway and such activation has been suggested as a possible mechanism of resistance to MEK inhibitors, researchers are also interested in determining whether inhibitors of the PI3K-Akt-mTOR pathway are effective in the treatment of *K-ras*-mutant CRC. Zhang *et al*[97] examined the effects of co-treatment with the mTOR inhibitor rapamycin and the MEK inhibitor PD89059 in *K-ras* mutant CRC cell lines. This combination inhibited cell proliferation with cell cycle arrest and induced apoptosis. Combinatory treatment with PI3K/Akt inhibitor and BRAF inhibitor showed synergistic growth inhibition in *B-Raf* mutated CRC cell lines that were resistant to a BRAF inhibitor[98,99]. Given the selectivity of BRAF inhibitors for the mutant form of *B-Raf*, the combination of BRAF inhibitors with PI3K/Akt pathway inhibitors is worthy of further investigation. EGFR or PI3K/Akt inhibitors combined with BRAF inhibition could be considered for individual cases of *B-Raf-*mutant CRCs with specific mechanisms of PI3K pathway activation, such as PTEN loss.

**CONCLUSION**

Personalized treatment of patients with mCRC based on genetic profiling of individual tumors is considered the future direction of cancer therapy. The important discovery that mutation of the *K-ras* gene is a predictor of resistance to EGFR monoclonal antibodies has brought this approach into clinical practice as an important innovation for the treatment of mCRC. However, this is only the first of a series of genetic predictors and an increasing number of molecular alterations have since been hypothesized to play a role in resistance to anti-EGFR drugs in CRC, including activating mutations in *N-Ras*, *B-Raf* and *PIK3CA*, and loss of expression of *PTEN*. These findings suggest that resistance to anti-EGFR agents involves a complex network of molecular alterations. Assessment of the effects of these alterations on the efficacy of new drugs that selectively target proteins introduces a new paradigm to clinical oncology. Because of the complexity and heterogeneity of molecular alterations in patients, the aim for the near future is the development of personalized anti-cancer drugs for the treatment of mCRC through definition of the mutation profile of key signaling genes in individual tumors. A comprehensive molecular characterization of mCRC and a better understanding of the functional interactions within the RTK-activated intracellular pathway will be necessary in order to select the most appropriate therapy for each individual patient.

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**Figure 1 Epidermal growth factor receptor and its downstream signaling in colorectal cancer.** Binding of ligands such as epidermal growth factor (EGF) to t epidermal growth factor receptor (EGFR) activates downstream Ras/ERK and PI3K/Akt pathways and regulates various physiological porcesses. EGFR mAbs (cetuximab and panitumumab) block activation of these pathways. Mutations of downstream molecules such as *Ras, PI3K*, or *Raf* are associated with resistance toEGFR mAbs in patients with metastatic colorectal cancer (mCRC).

**Table 1 Components of the epidermal growth factor receptor signaling pathway and their abnormalities in colorectal cancer**

CRC: Colorectal cancer.

**Table 2 RAF, MEK and PI3K/mTOR inhibitors presently in the status of clinical trial in colorectal cancer**