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## Liquid biopsy to detect resistance mutations against anti-epidermal growth factor receptor therapy in metastatic colorectal cancer

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### Abstract

Colorectal cancer (CRC) is a major cause of mortality worldwide, associated with a steadily growing prevalence. Notably, the identification of *KRAS*, *NRAS*, and *BRAF* mutations has markedly improved targeted CRC therapy by affording treatments directed against the epidermal growth factor receptor (EGFR) and other anti-angiogenic therapies. However, the survival benefit conferred by these therapies remains variable and difficult to predict, owing to the high level of molecular heterogeneity among patients with CRC. Although classification into consensus molecular subtypes could optimize response prediction to targeted therapies, the acquisition of resistance mutations to targeted therapy is, in part, responsible for the lack of response in some patients. However, the acquisition of such mutations can induce challenges in clinical practice. The utility of liquid biopsy to detect resistance mutations against anti-EGFR therapy has recently been described. This approach may constitute a new standard in the decision algorithm for targeted CRC therapy.

**Key Words:** Colorectal neoplasms; Precision medicine; Liquid biopsy; Cetuximab; Panitumumab

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**Core Tip:** Contemporary management of metastatic colorectal cancer patients with wild type *KRAS* includes the use of anti-epidermal growth factor receptor (EGFR) agents, such as cetuximab or panitumumab, as first-line treatment. However, a significant number of patients receiving this treatment show disease progression. Some of the relapses could be explained by the presence of acquired resistance mutations in *KRAS*. Liquid biopsy of circulating tumor cells or circulating cell-free DNA is expected to improve the management of patients undergoing anti-EGFR therapy.

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## INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide[1]. Disseminated disease (stage IV) with metastasis has been associated with poor prognosis, with a mean survival time of 15 mo[2]. The standard treatment for patients with metastatic CRC (mCRC) involves adjuvant chemotherapy with FOLFOX (leucovorin, 5-fluorouracil, and oxaliplatin) or FOLFIRI (leucovorin, 5-fluorouracil, and irinotecan). Furthermore, international guidelines recommend the analysis of *KRAS*/*NRAS* and *BRAF* mutations for targeted therapy[3,4]. Currently, the use of epidermal growth factor receptor (EGFR) inhibitor antibodies (anti-EGFR), such as cetuximab[5] or panitumumab[6], is recommended for patients with *KRAS* exon 2 wild-type (wt) mCRC. Both monoclonal antibodies exhibit a high affinity for the extracellular domain of EGFR; thus, they can prevent the ligand binding with EGFR[7]. Nevertheless, only 41% of patients with wt *KRAS* and left-sided colon disease reportedly attained partial or complete response to anti-EGFR treatments[8], as determined by RECIST criteria. The high level of variability in patient responses could be explained by the molecular and genomic variability of malignant colorectal neoplasms[9]. This heterogeneity could be explained by the consensus molecular subtype classification, which utilizes a transcriptomic approach to characterize the molecular heterogeneity of CRC[10]. This approach has opened new horizons by applying a novel classification to explain the distinct responses to conventional and targeted therapies in mCRC[11]. In addition to the heterogeneity of the primary tumor, the application of targeted therapies can lead to the selection of clonal tumor cells that acquire resistance mechanisms[12,13]. The emergence of activating *KRAS* mutations is a well-known (but not unique) mechanism of resistance to anti-EGFR therapy. For example, a retrospective analysis of the FIRE-3 clinical study (bevacizumab plus FOLFIRI or cetuximab plus FOLFIRI as first-line treatment for mCRC) has reported that a group of cetuximab-treated patients acquired activating mutations[14]. Furthermore, whole-exome sequencing studies have revealed that treatment with chemotherapy and cetuximab can be associated with a mutational signature (known as SBS17b) driving mutations in *KRAS*/*NRAS* and *EGFR* genes, resulting in resistance against this targeted therapy[15].

In real-world clinical settings, given that several patients are not considered suitable candidates for metastatic biopsies, it has been suggested that liquid biopsy could play a role in the early detection of mutations capable of inducing resistance to targeted therapies. Liquid biopsy is a recently described method that involves the analysis of genetic material from various sources, primarily blood (but also from urine, pleural fluid, and ascites). This method affords information on mutations and alterations in the copy number of genes related to the oncogenic process[16]. Several types of liquid biopsies are available, and the most widely used strategies involve the analysis of circulating tumor cells (CTC), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs) or exosomes, exhibiting both advantages and disadvantages[17]. In patients with mCRC, a high correlation has been noted between the primary metastatic tumor sample and ctDNA, approaching approximately 96.15% concordance for the analysis of *KRAS*, *NRAS*, and *BRAF*[18]. The objective of this review was to evaluate the role of liquid biopsy in the early identification of mutations that induce resistance to cetuximab or panitumumab therapy.

## ADVANCES IN LIQUID BIOPSY DETECTION TECHNOLOGY

Liquid biopsy requires technology capable of extracting tumor genetic material (DNA or RNA) from the blood, along with a technique that can quantify and characterize the molecular sequence. Nucleic acids can be detected by polymerase chain reaction (PCR)-based techniques or next-generation sequencing (NGS)[19]. The advantages of PCR-based techniques include their lower cost, shorter processing time,



and easier bioinformatics analysis than NGS techniques[20]. Disadvantages of PCR techniques include the selection of a prior bound study target and the difficulty in examining rare genetic alterations[21].

Advances in PCR techniques have allowed the development of digital PCR and subsequent evolution toward more advanced technologies such as droplet digital PCR (ddPCR) and Beads, Emulsion, Amplification, Magnetics (BEAMing) digital PCR. Both technologies employ digital PCR principles, which involve sample division or partitioning, where each partition occurs *via* independent reactions. Subsequently, a digital system allows fluorescence quantification in each partition, and combining the value of each partition affords a final quantification of molecules of interest[22]. In ddPCR, sample reactions occur within water-in-oil droplets, which act as a system of encapsulated molecules, where millions of PCR reactions can be simultaneously quantified[22,23]. The BEAMing technique involves digital PCR in emulsions combined with flow cytometry to quantify DNA molecules. In emulsions, DNA molecules and primers are attached to magnetic beads. Subsequently, amplified fragments are recovered by magnets and recognized by flow cytometry to measure the DNA of interest[24].

NGS techniques are based on massively parallel sequencing of selected or unselected genes; thus, millions of DNA sequences can be read simultaneously[25]. One main advantage of NGS is its ability to detect new mutations or mutations that rarely appear[22]. In addition, NGS offers high sensitivity and specificity for mutation detection; however, it exhibits considerable variability, ranging from 0.1% to 1%, depending on the technique or platform used[26].

Using liquid biopsy, tumor DNA can be obtained from various sources, including ctDNA, CTC, and EV, found in the blood of patients with cancer. Cells normally release nucleotides into patient blood. This genetic material can be isolated and is known as cfDNA. ctDNA is a part of cfDNA derived from tumor cells and can harbor mutations, amplifications, and epigenetic modifications associated with cancer[27]. CTCs are rare tumor cells in the blood that originate from solid tumors or metastases. Enrichment processes allow the elimination of leukocytes from the blood and CTC selection to extract the genetic material to be investigated[28]. Finally, EVs or exosomes are vesicles in the blood and contain DNA, mRNA, or miRNA modulating receptor cells[29].

Advances in methods and technologies for attaining genetic material are expected to complement the limitations of tissue or metastasis biopsies to improve patient prognosis[30].

## LIQUID BIOPSY FOR THE EXAMINING ANTI-EGFR RESISTANCE MUTATIONS

### ***Frequency of appearance of resistance in the EGFR pathway***

The EGFR receptor is a tyrosine kinase receptor, which, when ligand bound, activates the RAS, RAF, MEK, and ERK pathways[31]. The acquisition of activating mutations in any component of this pathway has been associated with oncogenesis[32]. Initial studies have focused on describing mutations in the *KRAS* oncogene in patients who relapsed following anti-EGFR therapy. Mutations in *KRAS*, a member of the small GTP-binding protein family, have been the focus of in-depth study, as the wt *KRAS* genotype is an indicator for anti-EGFR therapy. In a small number of patients with mCRC presenting disease progression, *de novo* mutations in *KRAS* measured by liquid biopsy[33] reached 38% (9/26). Reportedly, 40% of patients with mCRC exhibit *KRAS* mutations at diagnosis, most frequently in codons 12, 13, 61, and 146[34]. Mutations in codons 12 and 13 alter the position of the *KRAS* catalytic site at codon 61, reducing GTP hydrolysis and maintaining protein activity, even in the absence of a ligand[35, 36]. These activating mutations can induce cellular proliferation and suppress apoptosis[34]. Numerous theories have been proposed to clarify how anti-EGFR antibodies allow the acquisition of resistance mutations. For example, cell culture studies have revealed that prolonged exposure to anti-EGFR treatment allows the survival and selection of clones harboring *KRAS* mutations[37,38]. In addition, it is postulated that *de novo* mutations in resistance genes can be generated by genomic instability in cancer[35]. Furthermore, it has been proposed that the same therapeutic drugs can induce mutagenesis[39]. For example, patient studies have revealed that anti-EGFR treatment can induce a distinctive mutational signature, SBS17b, with preferential mutations in *KRAS* Q61H[15], which is consistent with cell culture studies demonstrating anti-EGFR treatment-induced mutagenesis[40].

The acquisition of resistance mutations in *KRAS* is one of the most frequent mechanisms reported in liquid biopsy studies. In a small study, 4 of 11 patients with wt *KRAS* treated with anti-EGFR antibodies acquired *KRAS* mutations, as determined by ddPCR of ctDNA. In addition, mutations in other components of the EGFR pathway, such as *BRAF*, *MET*, and *ERBB2*, were detected in three patients[41]. These results were replicated in a study by Vitiello *et al*[18] (2019), in which 10 new *KRAS* mutations were identified by automated quantitative reverse-transcription PCR in the ctDNA of 30 mCRC patients with wt *KRAS* receiving anti-EGFR therapy. In a further study using the BEAMing method, analysis of ctDNA revealed that 7 of 34 patients with wt *KRAS*, who were treated with anti-EGFR, developed resistance mutations, mainly in *KRAS* codons 12, 13, and 61[42]. Similarly, a follow-up program using the same methodology showed that, among 31 patients with wt *KRAS* tumor tissue receiving anti-EGFR treatment, 5 presented mutations in *KRAS* and 3 in *NRAS*[43]. Furthermore, an analysis of 62 patients with mCRC treated with cetuximab or panitumumab revealed 27 resistance mutations in *KRAS* and 5 mutations in *EGFR* (detected in plasma); mutations in codons 12 and 61 of *KRAS* were the most



common. Interestingly, the authors reported that the longer EGFR inhibitors were discontinued, the more the allelic frequency of these mutations detected in plasma tended to decrease[44]. Finally, an NGS study of ctDNA demonstrated that 69% of 42 patients treated with anti-EGFR had mutations or amplifications in *KRAS*, with the *KRAS* Q61H mutation (exon 2) detected in 52% of patients. Extending the analysis to other elements of the EGFR pathway, 91% of patients showed alterations in several pathway components, such as *NRAS*, *BRAF*, *MAP2K1*, *ERBB2*, *MET*, and *KIT* mutations or extensions, with an average of five alterations *per* patient for these genes[45]. Mutations conferring resistance to anti-EGFR are frequent, specifically in *KRAS/NRAS*, estimated to account for approximately 30%–89% of patients with mCRC (Table 1).

### **Prognosis associated with the appearance of anti-EGFR resistance mutations**

In addition, the prognostic utility of detecting resistance-acquired mutations during anti-EGFR therapy has been examined. Yamada *et al*[46] (2020) detected 20 acquired mutations in *RAS*, *BRAF*, or *EGFR* genes in ctDNA of 30 patients with mCRC treated with FOLFOX or FOLFIRI plus anti-EGFR. The authors reported that patients who developed measurable mutations in ctDNA had a worse prognosis for progression-free disease (PFS) than those with wt *RAS*. Follow-up analysis of patients with chemotherapy-refractory mCRC from the ASPECCT clinical trial[47] treated with panitumumab alone (conducted by liquid biopsy) revealed that 32% of 162 patients developed mutations in *RAS*. Mutations were found to primarily emerge in *KRAS* codons 2, 3, and 4 and less frequently in exon 2 of *NRAS*[48]. In contrast to previous studies, no significant differences were detected in patients with emerging *RAS* mutations in terms of PFS, overall survival (OS), or objective response rate. Subsequently, in the same cohort of patients, the authors found that the allelic frequency of resistance mutations in EGFR pathway genes, including *KRAS*, may be more closely associated with worse prognosis in panitumumab-treated patients[49]. These results are consistent with those of another study examining patients with wt *KRAS* CRC undergoing treatment with cetuximab or panitumumab; the emergence of mutations in *KRAS*, *NRAS*, or *BRAF* resulted in worse OS when compared with patients without mutations in these genes, as determined by analyzing CTC [hazard ratio (HR): 0.60, 95% confidence interval (CI): 0.40–0.91,  $P = 0.0028$ ], but not when ctDNA liquid biopsy was used to analyze the same cohort (HR: 0.80, 95%CI: 0.59–1.33,  $P = 0.088$ )[50]. In summary, growing evidence indicates that the detection of mutations, as well as allelic frequency, can be linked to the prognosis of mCRC.

### **Importance of timing for anti-EGFR treatment and emergence of resistance mutations**

It has been suggested that once disease progression is detected during anti-EGFR treatment, liquid biopsy can be used to evaluate the timing of reintroducing therapy[51]. This concept is known as rechallenge, whereby a period without treatment (such as anti-EGFR therapy) is followed by re-initiation of prior therapy, despite knowledge regarding the potential emergence of resistance mutations [8]. In a meta-analysis of patients who exhibited prior evidence of anti-EGFR benefits and rechallenge with anti-EGFR treatment (with a strategy of assessing *RAS* status by ctDNA liquid biopsy), up to 46% of patients converted from wt to mutant *RAS* following exposure to anti-EGFR treatment. Patients who maintained wt *RAS* before rechallenge had a better prognosis than those with a *de novo* *RAS* mutation [52]. Therefore, based on evidence suggesting a potential benefit in patients who maintain wt *RAS* prior to rechallenge, strategies have been proposed for patients who exhibit acquired resistance mutations in *RAS* following anti-EGFR treatment. Growing evidence indicates that resistance mutations decay over time after withdrawing anti-EGFR treatment; thus, withdrawing drug therapy eliminates the selective pressure on clones harboring resistance mutations[44]. An exploratory study of patients with wt *KRAS/BRAF* who acquired *RAS* or *EGFR* mutations during the course of anti-EGFR treatment showed that the frequency of mutant alleles decayed exponentially after discontinuing anti-EGFR treatment, with a mean of 4.4 mo[53]. In a retrospective cohort of 80 patients rechallenged after a longer interval, the authors reported a superior prognosis in terms of overall response[53]. Thus, considering the dynamics of the decay of clones with resistance mutations after treatment suspension, clinical studies have been proposed to corroborate the clinical utility of rechallenge therapies. For instance, it has been speculated that patients who previously progressed to chemotherapy and anti-EGFR antibodies could undergo second-line chemotherapy without anti-EGFR; if they progress, anti-EGFR rechallenge could then be performed based on *KRAS* allele frequency measurement[54]. This has been proposed in the REMARRY and PURSUIT phase II clinical trials; these studies suggested the reintroduction of FOLFIRI and panitumumab (which have an allelic frequency < 0.1% for mutated *KRAS*), allowing at least 4 mo without anti-EGFR administration[55]. Therefore, biopsies are not only useful for detecting resistance mutations, but could help determine the timing of treatment reintroduction once resistance-inducing mutations have declined.

**Table 1** Frequency of acquired KRAS resistance mutations in patients with stage IV colorectal cancer treated with cetuximab or panitumumab

Ref.	<i>n</i> wt KRAS patients at baseline	Analysis technique	Mutations or amplifications in KRAS/NRAS	Most frequent mutations
Vitiello <i>et al</i> [18], 2019	30	ctDNA/RT-qPCR	10 (30%)	KRAS Q61x (4) KRAS G12x (3)
Diaz <i>et al</i> [33], 2012	24	ctDNA/BEAMing	9 (36%)	KRAS G12x (9)
Pietrantonio <i>et al</i> [41], 2017	11	ctDNA/ddPCR	4 (36%)	KRAS Q61H (2)
Vidal <i>et al</i> [42], 2017	18	ctDNA/BEAMing	7 (39%)	KRAS G12x (5) NRAS Q61x (3)
Morelli <i>et al</i> [44], 2015	62	ctDNA/BEAMing	27 (43%)	KRAS G12x (10) KRAS Q61x (9)
Strickler <i>et al</i> [45], 2018	42	ctDNA/NGS DNAseq	26 (62%)	KRAS Q61H (22) KRAS G12A (5)
Yamada <i>et al</i> [46], 2020	19	ctDNA/ddPCR	16 (84%)	KRAS Q61H (10) KRAS G12V (9)
Kim <i>et al</i> [48], 2018	164	ctDNA/NGS DNA seq	53 (32.3%)	KRAS exon 3 (A59x o Q61x) (20)
Takayama <i>et al</i> [75], 2018	25	ctDNA/ddPCR	9 (36%)	KRAS Q12S (5) KRAS Q12D (4)

ctDNA: Circulating tumor DNA; ddPCR: Droplet digital PCR; NGS: Next-generation sequencing; BEAMing: Beads, Emulsion, Amplification, Magnetics.

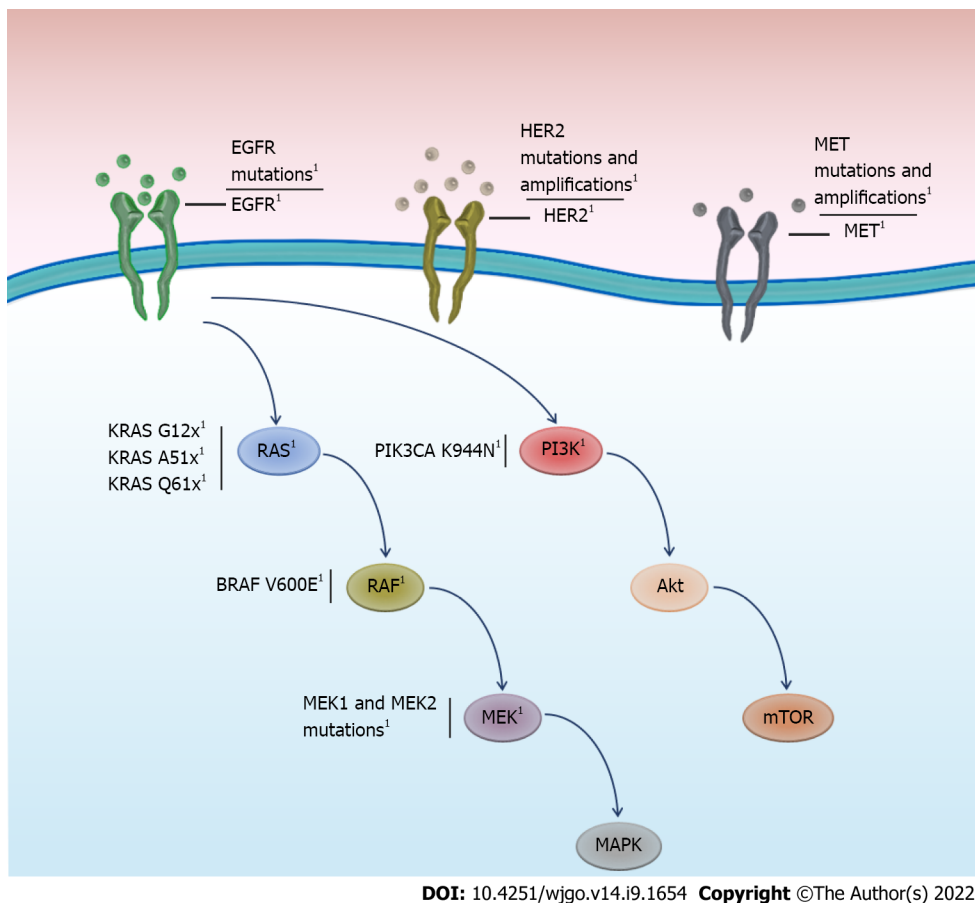
## FUTURE PERSPECTIVES

### Beyond KRAS/NRAS mutations

Resistance mutations to anti-EGFR treatment are frequent, particularly in *KRAS*, estimated to range between 30 and 89% (Table 1) in patients with mCRC. Although resistance mutations in *KRAS* are most frequent, mutations or amplification of other genes in the EGFR pathway, such as *ERBB2*, *MEK*, *BRAF*, and *MAP2K*, could also cause or contribute to anti-EGFR treatment resistance (Figure 1). Basic studies using patient-derived xenograft models, where the acquisition of natural resistance by chronic cetuximab exposure is reproduced, have reported the emergence of driver mutations in *EGFR*, *KRAS*, *MEK1*, and *MEK2*[56]. These results have been documented in real-world clinical settings, where patients were prospectively followed up by liquid biopsy. For instance, acquisition of *MET* amplification was frequent in wt *KRAS* mCRC (22.6%; 12/54 patients) that showed disease progression after anti-EGFR treatment, suggesting a possible mechanism of resistance[57]. Furthermore, a phase II clinical study proposed using a *MET* inhibitor to counteract the acquired resistance to anti-EGFR therapy. Tivantinib and cetuximab were administered to patients with histological evidence of *MET* overexpression. Although the combination did not afford superior benefit in patients, it was suggested that it might be more beneficial in patients with *MET* amplification[58]. Mutations acquired in *PIK3CA* (detected in ctDNA) could also induce resistance, based on analyzing a patient cohort with disease progression following cetuximab treatment[59]. A recent study suggested that the fusion of genes such as *FGFR2*, *FGFR3*, *RET*, *ALK*, *NTRK1*, and *ROS1* could emerge during anti-EGFR treatment; in particular, fusions involving *FGFR3* or *RET* could contribute to resistance to anti-EGFR therapy[60]. This finding allows the possibility of establishing liquid biopsy molecular panels to detect mutations causing resistance (beyond *KRAS*), which need to be validated in studies examining patients with mCRC undergoing anti-EGFR therapy.

### ERBB2/HER2

HER2 is a tyrosine kinase receptor and member of the HER/ERBB receptor family that includes EGFR (HER1), HER3, and HER4[61]. HER2/ERBB2 activation induces cellular proliferation and activation of the RAS/RAF/ERK and PI3KCA/PTEN/AKT pathways[62]. Mutations or amplification of HER2/ERBB2 has been detected in various tumors. Although most HER2-based studies have primarily focused on breast cancer, the role of this receptor in mCRC has recently been described[63,64]. Previous



**Figure 1 Main acquired resistance mutations detected by liquid biopsy.** Key acquired resistance mutations are associated with the epidermal growth factor receptor (EGFR) pathway. Other mutations or amplifications in tyrosine kinase receptors, such as HER2/ERBB2 or MET, can potentially lead to resistance to anti-EGFR therapy. <sup>1</sup>Indicate acquired resistance mutations, as reported in previous studies. EGFR: Epidermal growth factor receptor.

*in vitro* and prospective patient studies have suggested that both the presence of mutations related to the active site of the receptor and *HER2* amplification are associated with a poor response to anti-EGFR therapy[62,63,65]. In addition, the acquisition of mutations in *HER2* may be an underlying mechanism of secondary resistance that can be detected early using liquid biopsy. In a liquid biopsy study, 1 of 11 patients who progressed on anti-EGFR treatment showed *HER2* amplification and simultaneous mutation of *KRAS*[41]. In a study evaluating ctDNA by NGS, one case of *HER2* amplification was identified in a series of 15 patients treated with cetuximab[66]. Nonetheless, a case-control study revealed that the presence of *HER2* amplification in patients with wt *KRAS* CRC (prospectively measured by ddPCR of ctDNA) was not associated with a worse prognosis when compared with those without *HER2* mutations. However, the number of cases of amplified *HER2* was markedly low (five cases) to establish meaningful conclusions[67]. A phase IB clinical study has proposed the use of neratinib (pan-ERBB kinase inhibitor) and cetuximab in patients who have progressed to anti-EGFR therapy[68]. This trial was based on the hypothesis that *HER2*-negative tumors acquire *HER2* amplification as a mechanism of resistance to anti-EGFR treatment; neratinib, an irreversible inhibitor of EGFR, HER2, and HER4, improved prognosis in this subgroup of patients[69]. Evidence of *HER2* amplification was reported in 6 of 16 patients (assayed by chromogenic immunohistochemistry of metastatic biopsies or by NGS in ctDNA). Importantly, combining cetuximab with 240 mg/day of neratinib was well-tolerated, with a low incidence of adverse side effects[68]. Overall, current evidence from clinical models regarding the detection of acquired mutations in *HER2/ERBB2* is at an early stage, although this gene represents an interesting potential therapeutic target in patients who develop *HER2* amplification during anti-EGFR treatment.

#### **Toward liquid biopsy implementation in daily clinical practice**

Liquid biopsies for monitoring anti-EGFR resistance mutations have not been performed in routine medical practice. Real-world studies on liquid biopsy programs indicate that the application of these techniques can effectively alter the management of patients with colon cancer[43]. However, implementing these programs can pose challenges, including the high cost associated with these methods (PCR-based or NGS) and the lack of reimbursement[70], lack of cut-off values for detecting mutations, and absence of monitoring protocols[71].

Therefore, it is necessary to establish protocols for the frequency of taking liquid biopsies, as well as their implications for clinical patient management. Clinical studies are currently being conducted to standardize the frequency of sampling and interpretation of results. Two prospective studies have attempted to establish the prognostic value of liquid biopsy protocols; both studies including periodic three-monthly ctDNA analyses and clinical follow-up in CRC wt *KRAS* patients exposed to 5-fluorouracil regimens plus anti-EGFR antibodies[72,73]. Finally, current international guidelines, such as ESMO, have concluded that although there is insufficient evidence to recommend follow-up with liquid biopsy, such analysis could be useful for detecting secondary resistance to anti-EGFR[4]. In contrast, the Japanese Society of Medical Oncology clinical guidelines recommend the use of liquid biopsy because of its usefulness in monitoring anti-EGFR therapy[74].

## CONCLUSION

Based on current evidence, liquid biopsy could be developed as an innovative tool for managing patients with mCRC who receive anti-EGFR therapy. *De novo* *KRAS* mutations are one of the most commonly described mechanisms of acquired resistance and are associated with poor outcomes. However, establishing panels beyond *KRAS*, including genes related to the EGFR pathway, is crucial, given that such genes also potentially contribute to anti-EGFR resistance. Adequate strategies are needed to integrate liquid biopsy for the early detection of clinical progression of mCRC in patients undergoing anti-EGFR therapy. Future clinical studies will advance the routine use of liquid biopsy as a tool for reaching clinical decisions that benefit patients.

## FOOTNOTES

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