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REVIEW

KRAS mutation testing in metastatic colorectal cancer

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Abstract

The *KRAS* oncogene is mutated in approximately 35%-45% of colorectal cancers, and *KRAS* mutational status testing has been highlighted in recent years. The most frequent mutations in this gene, point substitutions in codons 12 and 13, were validated as negative predictors of response to anti-epidermal growth factor receptor antibodies. Therefore, determining the *KRAS* mutational status of tumor samples has become an essential tool for managing patients with colorectal cancers. Currently, a variety of detection methods have been established to analyze the mutation status in the key regions of the *KRAS* gene; however, several challenges remain related to standardized and uniform testing, including the selection of tumor samples, tumor sample processing and optimal testing methods. Moreover, new testing strategies, in combination with the mutation analysis of *BRAF*, *PIK3CA* and loss of *PTEN* proposed by many researchers and pathologists, should be promoted. In addition, we recommend that microsatellite instability, a prognostic factor, be added to the abovementioned concomitant analysis. This review provides an overview of KRAS biology and the recent advances in *KRAS* mutation testing. This review also addresses other aspects of status testing for determining the appropriate treatment and offers insight into the potential drawbacks of mutational testing.

Key words: *KRAS*; Epidermal growth factor receptor; Metastatic colorectal cancer; Testing status; Biomarker

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide. In the United States, approximately 102 900 cases of colon cancer and 39 670 cases of rectal cancer were diagnosed in 2010, and approximately 51 370 patients died of CRC in the same year, accounting for about 9% of all cancer deaths[1]. With the emergence of two anti-epidermal growth factor receptor (EGFR)-targeted antibodies, cetuximab (Erbitux) and panitumumab (Vectibix), the treatment of metastatic CRC has entered into the era of personalized treatment. Of the two antibodies, one is a human-mouse chimeric IgG1 monoclonal that was approved by the United States Food and Drug Administration (FDA) in 2004 as a second-line treatment of CRC; the other is a human IgG2 k monoclonal antibody that was approved by the FDA as a third-line drug in 2007. However, EGFR, the target of these drugs, which is overexpressed in approximately 80% of colorectal carcinomas, failed to predict a therapeutic response when used clinically[2,3]. Therefore, downstream signaling effectors were sought to help predict the efficacy of anti-EGFR treatment. The *KRAS* gene, which has

been extensively studied for more than three decades, has been demonstrated to be a strong negative predictive biomarker to indicate whether a CRC patient will respond to anti-EGFR treatment. As the target treatment may also be toxic and expensive, *KRAS* mutation status detection has become a crucial diagnostic factor for treating metastatic CRC patients.

KRAS GENE AND ITS ROLE IN EGFR SIGNALING

The *RAS* gene was initially identified as a viral gene homologous to the transforming gene from the Kirsten rat sarcoma virus[4,5]. Mutations in *RAS* are found in approximately 30% of all human cancers, making it one of the most commonly mutated genes in cancer[6]. The KRAS protein, also called p21, is a member of the *Ras* superfamily of proteins, is located on human chromosome 12 and encoded by 189 amino acids, and contains four coding exons and a 5' non-coding exon[7]. KRAS is a membrane-anchored guanosine triphosphate/guanosine diphosphate (GTP/GDP)-binding protein and is widely expressed in most human cells. As a small GTPase (GTP cleaving enzyme), KRAS is involved in intracellular signal transduction and mainly responsible for EGFR-signaling activation. The exchange of the active GTP-bound state and the inactive GDP-bound state is tightly controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors[8]. Under normal physiological conditions, upstream signals activate wild-type *KRAS* by promoting the exchange of bound GDP for GTP. This process is transient because of GAP-mediated GTP hydrolysis. However, this process becomes altered when the *KRAS* gene is mutated.

Mutant *KRAS* is found in about 35%-45% of CRCs[9-15], and codon 12 and 13 are two hotspots, which account for about 95% of all mutation types, with approximately 80% occurring in codon 12 and 15% in codon 13. Other mutations in codons 61, 146 and 154 occur less frequently in CRC, accounting for 5% of all mutation type[16]. Referring to the Catalogue of Somatic Mutations in Cancer Database, more than 5000 mutations have been found in the *KRAS* gene in CRC samples.

KRAS mutations are almost single nucleotide point mutations as reported, and the most common patterns are G12D, G12A, G12R, G12C, G12S, G12V and G13D. In the codon 12 mutation, p.G12D, pG12V is the most frequent, and in codon 13, the substitution of glycine for aspartate (p.G13D) is the most frequent[17].

These mutations impair the intrinsic GTPase activity of KRAS and prevent GAPs from promoting GTP hydrolysis by KRAS, therefore causing KRAS proteins to accumulate in the GTP-bound, active form. In this manner, mutant *KRAS* results in a constitutively active GTP-bound state and the activation of downstream proproliferative signaling pathways[18,19]. Therefore, *KRAS* mutations play a critical role in human tumorigenesis and are the most prevalent in pancreatic, thyroid, colorectal and lung cancers.

SIGNIFICANCE OF *KRAS* MUTATION TESTING

KRAS as a prognostic factor

It has been suggested that prognostic and predictive factors should be clarified; the former (including traditional clinical markers like lymph node involvement, the histological grade of the tumor, and molecular biomarkers, *etc*.) often refers to the outcome of the natural history of the tumor, while the latter predicts the response to the therapies. Until recently, the prognostic value of *KRAS* mutation was in dispute. Two canonical trials have demonstrated that the *KRAS* mutation may be prognostic of treatment outcomes for patients with CRC. The Kisten Ras in Colorectal Cancer Collaborative Group Study (RASCAL study)[20], with 2721 patient samples collected from 13 different nations, indicated that the presence of a *KRAS* mutation increased the risk of recurrence and death, especially in a guanine (G) to thymine (T) mutation. Moreover, the expanded RASCAL Ⅱ study suggested that the prognostic role of the *KRAS* mutation, limited only to a glycine to valine mutation, was found in 8.6% of all patients and had a statistically significant effect on failure-free survival [P = 0.004, hazard ratio (HR) 1.3] and overall survival (OS) (P = 0.008, HR 1.29)[21]. However, in a translational study of PETACC3[22], a randomized phase Ⅲ trial showed that the *KRAS* mutation status does not have major prognostic value in stages Ⅱ and Ⅲ colon cancer. The difference in results may be largely due to the difference in sample size. The results from other trials are also not consistant[23].

KRAS as a predictive factor

Because *KRAS* is the most frequently mutated factor downstream of the EGFR signaling pathway, it was considered a candidate molecular biomarker for anti-EGFR therapy. In 2006, for the first time, the predictive value of *KRAS* was validated in a study by Lièvre *et al*[24] in which the *KRAS*-mutated patients showed no response to cetuximab and had a poorer OS compared with the wild-type *KRAS* patients. Later, a series of single-arm studies confirmed this result[25-29]. Then, not only cetuximab but also panitumumab were demonstrated to only be effective for wild-type *KRAS* patients[30,31]. These trials demonstrated that the outcomes of patients with wild-type *KRAS* were clearly better than those of the *KRAS*-mutant patients, although these were all retrospective analyses. The publication of two large, multicenter, randomized phase Ⅲ clinical trials unequivocally demonstrated the predictive value of *KRAS* for anti-EGFR therapy (Table 1). In these two trials, panitumumab or cetuximab *vs* best supportive care (BSC) was given to patients with chemorefractory CRC compared with BSC alone. Amado *et al*[10] demonstrated that the response rate of panitumumab was 17% and 0% for the wild-type *KRAS* group and the mutant group, respectively (P < 0.0001). In addition, when combined with chemotherapy [5-fluorouracil, leucovorin and irinotecan (FOLFIRI) or 5-fluorouracil, leucovorin and oxaliplatin], anti-EGFR

Table 1 Predictive value of *KRAS* for anti-epidermal growth factor receptor therapy in metastatic colorectal cancer

Reference	Regimen	Treatment line	Phase	*n*	Mutation status (%)	Method	Remarkable results
Monotherapy							
Karapetis *et al*[9], 2008	Cetuximab *vs* BSC	Chemotherapy refractory	III	394	42.3	Sequencing	Cetuximab alone works on patient with WT KRAS tumors
Amado *et al*[10], 2008	Panitumumab *vs* BSC	Chemotherapy refractory	III	427	43	Allele-specific PCR (DxS, United Kingdom)	Panitumumab alone works on patient with WT KRAS tumors
Combined with chemotherapy							
Van Cutsem *et al*[11], 2009	Cetuximab + FOLFIRI, FOLFIRI	First-line CRYSTAL trial	III	540	35.6	PCR clamping and HRM (TIB MolBioL, Germany)	Cetuximab plus FOLFIRI, reduced the risk of progression of metastatic colorectal cancer
Bokemeyer *et al*[12], 2009	Cetuximab + FOLFOX, FOLFOX	First-line, OPUS trial	II	233	42	PCR clamping and HRM (TIB MolBioL, Germany)	Significantly increased ORR in patients with WT KRAS tumors
Peeters *et al*[13], 2010	Panitumumab + FOLFIRI FOLFIRI	Second-line	III	1083	45	Allele-specific PCR (DxS, United Kingdom)	Significantly improved PFS in patients with WT KRAS tumors
Douillard *et al*[14], 2010	Panitumumab + FOLFOX FOLFOX	First-line	III	1096	40	Allele-specific PCR (DxS, United Kingdom)	Significantly improved PFS in patients with WT KRAS tumors
Van Cutsem *et al*[15], 2011	Cetuximab + FOLFIRI, FOLFIRI	First-line	III	1063	37	PCR clamping and HRM (TIB MolBioL, Germany)	Significantly improved OS in patients with WT KRAS tumors

BSC: Best supportive care; WT: Wild type; ORR: Overall response rate; FOLFIRI: 5-fluorouracil, leucovorin and irinotecan; FOLFOX: 5-fluorouracil, leucovorin and oxaliplatin; PFS: Progression-free survival; OS: Overall survival; PCR: Polymerase chain reaction; HRM: High-resolution melting.

antibodies (cetuximab or panitumumab)-treated patients had a better response rate and progression-free survival (PFS) or OS alone in the wild-type *KRAS* group, regardless of the treatment line[11-15]. Recently, better OS (median, 23.5 mo *vs* 20.0 mo; HR 0.796, $P = 0.0093$) was found in the cetuximab plus FOLFIRI-treated wild-type *KRAS* patients compared with the FOLFIRI-treated KRAS-mutated patients[15]. According to a recent meta-analysis of 11 studies conducted between 1966 and 2010[32], the *KRAS* status and the adding of anti-EGFR antibodies to standard chemotherapy were closely related to PFS [95% confidence interval (CI): 57%-90%, $P = 0.005$] and response rate (95% CI: 8.22%, $P < 0.001$).

On the basis of these results, National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO) and European Medicines Evaluation Agency recommended testing for *KRAS* gene mutations in advanced CRC patients. The NCCN added *KRAS* testing to their 2009 clinical practice guidelines for colon and rectal cancers[33,34] and stipulated that only patients with wild-type (normal) *KRAS* genes should receive treatment with cetuximab (Erbitux) or panitumumab (Vectibix). The ASCO, in the same year, proposed a provisional clinical opinion (PCO)[35] demonstrating that testing for *KRAS* mutations should be performed prior to anti-EGFR monoclonal antibody therapy and that patients with KRAS mutations in either codon 12 or 13 should not receive this therapy as part of their treatment. This recommendation is slightly different from the NCCN guideline because the use of anti-EGFR therapy in the *KRAS*-mutated patients may be toxic.

KRAS TESTING STATUS

Frequency of testing

In a recent three cross-sectional survey performed in Europe, Latin America and Asia[36], physicians completed questionnaires on four patients per year. An analysis of 3800 samples per year showed that the *KRAS* testing frequency in metastatic CRC patients increased from 3% in 2008 to 47% in 2009 and 69% in 2010. It appears that the importance of *KRAS* mutation testing has become progressively understood by physicians and oncologists. Because implementation of the testing in the clinical practice has begun, it is essential to identify testing performance, as there are no set criteria for the process of *KRAS* detection, i.e., the selection of tissue specimens, specimen preparation, the timing of testing and the best method.

External quality assessment

A *KRAS* external quality assessment protocol was established in 59 laboratories throughout eight different European countries[37]. In the first assessment round, the results were unsatisfactory. The samples, including unstained sections of 10 invasive CRC with a known *KRAS* mutation status, were tested by each laboratory using their own preferred method for histological evaluation, DNA isolation, and mutation analysis. The test results were centrally validated by one of two reference laboratories. Only 70% of the laboratories correctly identified the *KRAS* mutational status in all samples, and the reports often lacked essential information. In another quality assessment for *KRAS* testing in Italy, five CRC specimens with known *KRAS* mutations were sent to be tested in 59 centers[38]. The limit to pass the assessment was set at 100% true responses. Only two centers failed in both the first round and the second round of testing. In Canada, until recently, there has been no such quality assessment. However, a guideline was developed according to a Canadian consensus conference held in Montreal in April 2010, in which the expert group provided recommenda-

tions on *KRAS* testing in the treatment of CRC[39]. In the United States, there is currently no FDA-approved standardized test. However, the PCO provided recommendations to the *KRAS* testing clinics. In Asia, there has been no external quality assessment system as yet, and it is critical to fulfill this objective.

Mutation status

As reported in 2011, the *KRAS* mutation frequencies in Asia, Europe, Latin American were 24%, 36% and 40%, respectively ($P < 0.0001$)[36]. It is unclear why a lower incidence is observed in Asian patients. In China, *KRAS* mutations were detected in 33.3% (30/90) of the CRC tumor samples using the nucleotide sequence analysis method[40]. These results significantly correlated with the response rate and survival time of cetuximab-treated patients. The difference of mutation status may result from many aspects, such as the tissue, the percent of tumor cells, the extracted DNA quality, the testing methods and the testing target.

Testing target

Currently, in most of the *KRAS* detection methods, only mutations of codon 12 or 13 are certified as informative for selecting non-responders to the anti-EGFR treatment in large clinical trials[15]. Therefore, mutation analysis of these sites is recommended. However, recent research has revealed new findings. Mutations in exons 3 and 4 are also effective in predicting the efficacy of EGFR-antibodies[41,42]. Codon 61 was found to account for 2% of all *KRAS* mutations and, similar to some of the codon 12 mutations, had predictive value[43]. Therefore, codon 61 may be useful in *KRAS* mutation testing. In contrast, not all mutations in codon 13 appear to be informative. In a recent analysis, cetuximab surprisingly worked on patients with chemotherapy-refractory CRC with p.G13D-mutated tumors, and these patients have a longer overall and PFS compared with those with the *KRAS*-mutated tumors[44]. Therefore, efforts are still required to confirm the importance of various mutations of the *KRAS* gene.

Sample selection

The most widely used tissue for *KRAS* testing is formalin-fixed paraffin-embedded (FFPE) tissue blocks[45], which are easy to obtain and convenient to preserve. However, DNA extracted from FFPE is time consuming and may be of poor quality, which can also result in false-positive or false-negative results due to an incomplete tissue fixation or tissue overfixation. Another specimen type is frozen tissue. Studies that compared the mutation detection rates in frozen and FFPE samples from the same tissue have found that the mutation rate in frozen samples is higher than that detected in FFPE samples[46]. The use of frozen tissue is suggested to be the gold standard for analysis, but the associated expense and technical difficulty of using frozen tissue make this method unsuitable for routine testing. In contrast, a high concordance was observed between primary tumors and metastatic locations (91.7%-96.4%)[47-49]. Therefore, the *KRAS* status in a primary site can be used for selecting patients who would benefit from anti-EGFR therapy. However, *KRAS* status can be heterogeneous within a primary tumor, and thus, different parts of such tumors should be examined to accurately predict the *KRAS* status in metastatic lesions.

Beyond the selection of tissue, other choices, such as peripheral blood, have been studied. Yen *et al*[50] detected circulating tumor cells with *KRAS* oncogenes using membrane arrays; *KRAS* mutations were identified in 39.5% (30/76) of peripheral blood samples, which is similar to that in tumors (43.4%). According to a review concerning the validation of *KRAS* mutation testing in CRC blood samples which summarizes the studies that detect *KRAS* status using tissue or plasma/serum[51], a positive *KRAS* mutation in plasma or serum suggests a *KRAS* mutation in the tumor whereas the absence of a *KRAS* mutation in the plasma or serum does not necessarily prove a lack of a similar mutation in the CRC tumor tissue. Further studies are needed in this field.

Methods

A number of methods can be used in *KRAS* mutation testing, with different sensitivity, turnaround time, and cost. In the NCCN guideline or ASCO PCO, no explicit method was assigned. Therefore, the use of assays worldwide is somewhat chaotic. In the Italian quality assessment for *KRAS* testing[38], five CRC specimens were sent to 59 centers, which were asked to use their own preferred method for DNA extraction and mutational analysis. Of these 59 centers, polymerase chain reaction (PCR) sequencing was the predominant method for mutational analysis, as 48 (81.3%) centers used this methodology. Among the remaining centers, 5 centers (8.5%) used pyrosequencing, 3 centers (5.1%) used Real-Time PCR (Therascreen kit), 2 centers (3.4%) used restriction fragment length polymorphism (RFLP) analysis and 1 center (1.7%) used the KRAS strip assay. In the United States, the amplification refractory mutation system was used by most laboratories[52].

The traditional methods used for mutation testing are hybridization and DNA sequencing. These methods are complex and time consuming. The emergence of polymerase chain reaction (PCR) sheds new light on this field. Currently, mutation testing methods are almost exclusively based on this technology, including PCR-based sequencing, high resolution melting analysis (HRMA), amplification refractory mutation system (ARMS), and cleaved amplification polymorphism sequence-tagged sites (PCR-RFLP).

Among these methods, DNA sequencing, also called Sanger sequencing or dideoxy sequencing, is considered the gold standard because this methodology analyzes the DNA sequence nucleotide by nucleotide and can identify all possible mutations in the analyzed *KRAS* gene segment, including base substitutions, insertions and deletions. However, this approach has a low sensitivity of about 20%, and is laborious and time consuming. An alternative approach to this methodology, i.e.,

Table 2 Methods used for *KRAS* mutation testing[45,55,60-62]

Method	Sensitivity (mutant/wild-type) (%)	Turnaround time	Main advantages	Main disadvantages
Sanger sequencing	20–30	Slow (4 d to 2 wk)	Detects all possible mutations, cost-effective	Insensitive, time consuming, open PCR system is easily contaminated
Pyrosequencing	5	Rapid	Detects all possible mutations, sensitive	Open PCR system is easily contaminated
Real-time PCR with HRMA	5	Rapid	Rapid, closed PCR system, detects all possible mutations (heterozygous and homozygous)	Occasionally difficult to distinguish between mutation types
Allele-specific real-time PCR	10	Rapid	Rapid, closed PCR system	Detects only the 7 most common mutations, requires more tissue for analysis compared with other methods
RFLP with sequencing	0.1	Slow (4 d to 2 wk)	Sensitive	Requires confirmation by sequencing, complicated
DxS (ARMS/S)	1	Rapid	Sensitive, time-saving	Expensive, detects specific mutations targeted by the designed primers
COLD-PCR with sequencing	1-2.5	Rapid	Sensitive, cost-effective, detects all possible mutations	-

ARMS: Amplification refractory mutation system; RFLP: Restriction fragment length polymorphism; PCR: Polymerase chain reaction; COLD-PCR: Coamplification at lower denaturation temperature PCR.

pyrosequencing, has a sensitivity proven to be approximately 5%-10% and has commercialized the detection of *KRAS* mutations; corresponding commercial kits, the PyroMark® (Qiagen, Valencia, CA, United States), have been developed[53,54].

Of the non-sequencing methods, ARMS[55], real-time PCR analysis with HRMA[56], RFLP[57] and allele-specific real-time PCR[58], most of which are based on real-time PCR technology, have been well studied in the past three years with Sanger sequencing as the reference, demonstrating the effectiveness and availability of these methods for *KRAS* status testing. A multicenter study[59], which evaluated six different *KRAS* mutation detection methods, including pyrosequencing, HRMA, dideoxy sequencing, and two commercial kits, showed a concordant *KRAS* status in 66/80 (83%) of frozen tissue samples and 71/74 (96%) of paraffin tissues using the five best performing assays. Each of the assays has its advantage and limitations, and as details have been described in previous publications, we have summarized some notable features in Table 2[45,55,60-62]. The HRMA assay, often based on real-time PCR, detects the mutant sequence through measuring changes in the melting of a DNA duplex with the aid of intercalating dyes. This method is fast and sensitive but has been reported to have a false-positive rate of 20%[63]. Therefore, this method requires sequencing confirmation and cannot show the concrete mutation pattern. The allele-specific Real-Time PCR and ARMS can only detect the limited mutation sites of the *KRAS* gene, which makes these methods less feasible in clinical practice. The ARMS-based commercial kit, TheraScreen® (DxS Ltd, Manchester, United Kingdom), however, has been widely used in laboratories[64]. This kit has a real-time PCR-based assay that combines the ARMS with Scorpion probes (seven probes for seven different mutations in *KRAS*), eliminating the need for post-PCR confirmation by direct sequencing, and is thought to be the most sensitive method until recently with a sensitivity of 1%[45].

Recently, more sensitive methods have been utilized in *KRAS* detection. One method is the PCR-clamp assay, and the other is coamplification at lower denaturation temperature PCR (COLD-PCR). The PCR-clamp assay utilizes mutation-specific hybridization probes and another wild-type-complementary peptide nucleic acid probe to suppress the amplification of the normal sequence and can detect less than 1% of the allele[65,66]. A commercial kit (KRAS LightMix) by TIB MolBiol (Berlin) uses this technology and a melting curve analysis and has been used in multicenter, phase Ⅲ clinical trials in which patients were treated with the anti-EGFR antibody, cetuximab[11,12,15]. COLD-PCR is another selective amplifying system that enriches the "minority alleles" from the mixed DNA sequences based on the lower melting temperature of mutant homoduplexes as compared with wild-type ones. Therefore, in COLD-PCR, the denaturation temperature is set at 80 ℃ whereas the denaturation temperature in conventional PCR is approximately 94 ℃. Using this principle, this technology does not require special equipment or reagents or time-consuming procedures. As a sensitive DNA enrichment method, COLD-PCR is often followed with HRMA or pyrosequencing. Mancini *et al*[67] demonstrated that COLD-PCR combined with HRM can improve the limit of detection of *KRAS* and *BRAF* mutations in CRC, increasing the percentage of mutated CRCs from 40% (47/117) to 48.7% (57/117) compared with traditional PCR and direct sequencing. In another study by Zuo *et al*[68], COLD-PCR combined with pyrosequencing detected all the mutations in 50 samples, including DNA extracted from either fresh or FFPE tissue specimens that were confirmed positive by conventional PCR, and the mutation detection sensitivity was certified as 1.5%.

In addition, COLD-PCR combined with HRMA

assay does not require expensive and time-consuming procedures; thus, in clinical settings, this procedure has the potential to be used to select those patients who are eligible for EGFR-targeted therapies.

Our recommendation

Currently, it is accepted that the DNA fragmentation caused by improper fixation, heterogeneous somatic *KRAS* gene mutations, and the influence of stromal cells can cause false-positive *KRAS* mutation testing results. Fortunately, the technique refinements and sufficient tissue selected can reduce this limitation. It is suggested that at least 300 tumor cells or 30 ng of template DNA are required for *KRAS* status analysis. However, the appropriate method to extend to the clinic is still unclear. Molinari *et al*[69] found that highly sensitive methods could improve the accuracy of predictions of anti-EGFR monoclonal antibody efficacy. Therefore, assay sensitivity when detecting *KRAS* mutations is a key issue for correctly analyzing tumor specimens. However, Carotenuto *et al*[70] demonstrated that in samples with more than 30% tumor cells, the DxS assay and PCR-sequencing, which are the most sensitive and non-sensitive methods, respectively, showed no difference in identifying *KRAS* mutations. Therefore, more effective and sensitive methods are required for inconclusive samples and those with a low number of tumor cells. Upon considering the sensitive detection methods, as previously described, pyrosequencing is a new, robust but expensive technology. The DxS assay (ARMS/S) is now widely used in clinical labs but can only detect the seven common mutations, and it is costly. COLD-PCR, which can enrich the mutant alleles, is considered a simple method that increases *KRAS* testing sensitivity. Therefore, we recommend the use of this assay combined with HRM or sequencing for determining *KRAS* status; although, this approach should be validated by further large sample studies.

CONCOMITANT ANALYSIS WITH OTHER FACTORS

Unfortunately, *KRAS* mutations account for approximately 35% of the nonresponsive patients that receive anti-EGFR treatment[35]. Therefore, using *KRAS* as a predictor of clinical outcomes is not always useful. These results have led researchers back to the molecular mechanisms of cetuximab and panitumumab resistance to find other powerful prognostic markers. *BRAF*, which is another member of EGFR signaling cascade, is located downstream of *KRAS* and is considered the most promising marker for predicting anti-EGFR treatment resistance apart from *KRAS* gene. *BRAF* mutations mainly occur at exon 15 with a frequency of approximately 5% to 10% and the common V600E pattern. It is notable that *BRAF* and *KRAS* mutations are mutually exclusive ($P < 10^{-6}$)[71]. Therefore, *BRAF* mutation analysis is recommended when the *KRAS* gene is the wild type. Di Nicolantonio *et al*[72] found in a retrospective study that none of the *BRAF*-mutated patients responded to cetuximab or panitumumab and that none of the responders carried *BRAF* mutations ($P = 0.029$). In addition, *BRAF*-mutated patients had a significantly shorter PFS ($P = 0.011$) and OS ($P < 0.0001$) compared with wild-type patients. On the basis of these results, the NCCN clinical guidelines in 2010 currently recommend *BRAF* mutational status assessment of metastatic CRC patients with a wild-type *KRAS* to guide the therapeutic use of cetuximab and panitumumab.

Apart from the *KRAS* and *BRAF* gene mutations, other genetic aberrations, such as *PIK3CA* and *PTEN*, were demonstrated to be helpful in predicting the resistance to anti-EGFR treatment[40,73]. In addition, many oncologists and pathologists have proposed that combining the analysis of these factors simultaneously will provide a clearer overall prognostic indication for EGFR inhibitor status. The recent data from a retrospective analysis demonstrated that when the loss of *PTEN* expression and mutations of *KRAS*, *BRAF* and *PIK3CA* are concomitantly ascertained, as many as 70% of the metastatic CRC patients can be identified as unlikely to respond to anti-EGFR therapies[74]. Therefore, CRCs lacking alterations in *KRAS*, *BRAF*, *PTEN* and *PIK3CA*, which may have the highest probability of response to anti-EGFR therapies, are defined as "quadruple negative"[74,75].

In addition, in a retrospective consortium analysis[43], the largest series to date according to our knowledge, the effects of *KRAS*, *BRAF*, *NRAS* and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal was studied. In total, 1022 tumor DNA samples were tested, of which 40.0% (299/747) harbored a *KRAS* mutation, 14.5% had a *PIK3CA* mutation, 4.7% had a *BRAF* mutation, and 2.64% *NRAS* mutation, and carriers of the four mutations had a lower response rate to the cetuximab plus chemotherapy treatment compared with those lacking any of the four mutations. A multivariate analysis also confirmed that if *KRAS* is unmutated, assessing the *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations provides additional information about patient outcomes. It is notable that while *NRAS* accounts for only 2.64% of these molecular alterations, this mutation is associated with unresponsiveness to panitumumab treatment.

It is obvious that *KRAS* mutational status analysis is insufficient for predicting the efficacy of anti-EGFR therapy, and adding the concomitant analysis of downstream factors can be helpful in selecting the correct patient for this personal treatment. In addition, we suggest that microsatellite inestability (MSI) be added to this concomitant analysis.

Microsatellite instability, defined as small deletions or expansions within short tandem repeats in tumor DNA resulted from the inactivation of the DNA mismatch repair system, has been found in up to 90% of the tumors of the hereditary nonpolyposis CRC and in approximately 20% of sporadic colorectal tumors[76,77]. Using a panel of 5 microsatellites recommended by the National

Cancer Institute, i.e., BAT 25 and BAT 26 (mononucleotide repeats), D2S123, D5S346 and D17S250 (dinucleotide repeats), CRC tumors are classified as MSI-high (MSI-H), MSI-low (MSI-L) and microsatellite stability (MSS), and the MSI-H was thought to indicate a more favorable prognosis[78]. However, with regard to predicting therapy response, the role of MSI is conflicting. Recently, some researchers have combined *KRAS* and MSI in their study[79] and found that both genes are prognostic of CRC. In another study[80], the combined analysis of specific *KRAS* and *BRAF* mutations, and microsatellite instability were used to identify prognostic subgroups of sporadic and hereditary CRC. As the result, 3 distinct prognostic subgroups were observed in univariate (P = 0.006) and multivariable (P = 0.051) analysis: group 1 consisted of patients with *KRAS G12D* or *G12V* or *BRAFV600E* mutations independent of MSI status; they had a poor survival time and suffered more patient deaths. Group 2 included patients with either wild-type *KRAS*/*BRAFV600E* or *KRAS* G13D mutations in the MSS/MSI-L tumors and had a more favorable outcome. Finally, the patients with MSI-H cancers and simultaneous *G13D* mutations were observed to have the worst outcomes. The survival times for groups 1-3 varied significantly (P = 0.006). Therefore, we recommend the concomitant analysis of *KRAS, BRAF, PIK3CA,* and *PTEN* combined with MSI, which can facilitate selecting the appropriate patients for anti-EGFR treatment while also indicating the outcome of CRC patients.

CONCLUSION

KRAS, an important member of the EGFR signaling cascade, can acquire activating mutations in codons 12 and 13 of exon 2 in approximately 35%-45% of the CRC cases, rendering EGFR inhibitors ineffective. Though the prognostic value of *KRAS* is conflicting, it is a promising predictive biomarker of personalized treatment. Numerous clinical trials have clarified the significant benefit of outcomes in patients with wild-type *KRAS* for anti-EGFR therapy, despite the treatment line. Therefore, *KRAS* status testing has been recommended by national organizations, including NCCN, American Society for Clinical Oncology and European Medicines Agency. In recent years, *KRAS* testing is administered with a high frequency; however, standards are desired worldwide, including the selection and processing of the tumor sample and the choice of the appropriate detection method, which may affect the accuracy of the testing results. COLD-PCR is a simple assay that can increase *KRAS* testing sensitivity by enriching the mutant alleles. This technology combined with HRM or sequencing is potentially useful in *KRAS* detection in a clinic practice. In addition, concomitant analysis with other factors, such as BRAF, PIK3CA, PTEN and MSI, is helpful in supporting *KRAS* as predictive and prognostic factors, but further efforts are needed prior to implementation.

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