

Extended *RAS* testing in metastatic colorectal cancer—Refining the predictive molecular biomarkers

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Abstract: Mutations of exon 2 of *Kirsten rat sarcoma viral oncogene homologue (KRAS)* (exon 2 codons 12/13) lead to constitutive activation of the EGFR (epidermal growth factor receptor) mediated signal transduction pathway and been shown to be a negative predictive biomarker for EGFR-directed monoclonal antibodies among patients with colorectal cancer (CRC). As selection of patients is very important for administration of anti-EGFR therapy, this lone biomarker has proved to be insufficient for selecting the appropriate patients as more patients lacking exon 2 *KRAS* mutation were resistant to anti-EGFR therapy. The results of various randomized clinical trials have confirmed the presence of other *RAS* mutation including additional *RAS* mutations (*KRAS* exons 3/4 and *NRAS* exon 1/2/3/4). Extended *RAS* analysis should be considered before initiating anti-EGFR therapy to patients of metastatic CRC. This can help in proper selection of patients leading to tailored individualistic treatment, decreasing cost of treatment and the adverse effects related to use of monoclonal antibody therapy. The new evidence is supporting the need to make ‘Extended *RAS*’ analysis essential before start of treatment with anti-EGFR monoclonal antibody therapy. Prior to this the Extended *RAS* testing should be standardized.

Keywords: Extended *RAS* analysis; metastatic colorectal cancer (CRC); monoclonal antibody therapy

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Introduction

According to the American Cancer Society, the latest records of year 2012 showed that colorectal cancer is the third most commonly diagnosed cancer and the third leading cause of cancer death among both men and women in USA (1). The management of this widely prevalent cancer has also been evolving from being non-specific to being patient and target specific in the recent past. As a step towards targeted treatment, epidermal growth factor receptor (EGFR) was validated as a therapeutic target for chemotherapeutic agents (2).

Various randomized controlled trials (RCTs) proved the beneficial effects of anti-EGFR monoclonal antibodies as monotherapy as well as in combination therapy among

patients with metastatic colorectal cancer (mCRC) in the last decade (3-7). Two anti-EGFR monoclonal antibodies (mAbs), cetuximab and panitumumab, were approved for use alone or with standard chemotherapy among patients with advanced CRC (8,9). As the mAbs are expensive and can be potentially toxic drugs, there was a need for proper selection of patients eligible for administration of the antibody based therapy. EGFR expression level was the first biomarker to be studied among patients likely to be prescribed anti-EGFR mAbs. But, no correlation could be established between the response to anti-EGFR mAbs and the EGFR expression levels (6,10). Later, an association between the occurrence of mutation of *KRAS* gene and the poor response with anti-EGFR mAbs was established (11). This was followed by the recommendation of testing of

mutational status of *KRAS* gene before initiation of therapy with anti-EGFR mAbs among patients of mCRC (12,13).

EGFR and RAS signaling pathway

EGFR, a tyrosine kinase receptor involved in signal transduction mechanism, is one of the important molecular targets for drug therapy (14). Binding of EGF or any other ligand to EGFR activates signal transduction via various pathways. These include the RAS-RAF-BRAF-MAPK (mitogen activated protein kinase) pathway or phosphatidylinositol 3-kinase (PI3K)-Akt or phospholipase C γ pathway (15). *RAS* is the most important superfamily of proteins, which includes mainly *KRAS* and *NRAS* proteins. *KRAS* is a guanosine triphosphate cleaving enzyme (GTPase). The signaling through *KRAS*-RAF-BRAF-MAPK pathway controls gene transcription, cell proliferation, apoptosis, angiogenesis, invasion and migration (16-18).

Although EGFR is a molecular target for anti-EGFR mAbs and is also over expressed among approximately 80% of CRCs, it could not be established as a predictive biomarker in the management of CRC (16,19). Positive EGFR protein expression proved to be a poor biomarker for response with anti-EGFR mAbs (18). Thus, other effectors in the downstream signal transduction pathway were evaluated for their predictive value. It was observed that mutation in *KRAS*, *NRAS*, *BRAF* or *PI3KCA* genes result in constitutive activation of signaling pathway. Approximately 30-50% CRCs carry a mutation at codon 12 or 13 of exon 2 of the *KRAS* gene, followed by mutations of *NRAS*, *PI3KCA* and *BRAF* (20,21). These mutations are responsible for constitutive activation of EGFR downstream pathways which disrupt the normal signaling pathway independent of EGFR (15,18). Mutations in *BRAF* lead to uncontrolled *BRAF* activation independent of EGFR and *RAS* (17).

KRAS mutant status as a predictive biomarker

After the approval of cetuximab and panitumumab for use among patients with mCRC, various studies demonstrated that these drugs were effective among patients with *KRAS* exon 2 wild type tumors only and not among those with *KRAS* exon 2 mutant tumors (22,23). The median progression free survival (PFS) and overall survival (OS) significantly improved among the *KRAS* exon 2 wild type

group with anti-EGFR antibody therapy when used either in monotherapy or combination therapy as compared to the basic support care group or standard chemotherapy regimen respectively (22,23). On the other hand, the *KRAS* exon 2 mutant group did not show any difference in efficacy with the addition of anti-EGFR mAbs as compared to the standard chemotherapy regimen (22-25). In addition, somewhat unexpected detrimental effects were observed in the mutant *KRAS* groups in the PRIME (panitumumab randomized trial in combination with chemotherapy for metastatic colorectal cancer to determine efficacy) and OPUS (oxaliplatin and cetuximab in first-line treatment of mCRC) studies (26,27). Both prospective and retrospective analysis of the clinical studies concluded that mutation of codon 12 or 13 of exon 2 of *KRAS* is a negative predictive biomarker for therapy with anti-EGFR antibody therapy (11,22-27).

This led to the recommendation for routine *KRAS* exon 2 mutational testing. The American Society of Clinical Oncology and National Comprehensive Cancer Network (NCCN) recommended that all patients with mCRC who are candidates for anti-EGFR antibody therapy should have their tumor tested for *KRAS* mutations. If *KRAS* mutation in codon 12 or 13 is detected, then patients with mCRC should not receive anti-EGFR antibody therapy as part of their treatment due to the predicted lack of response (12,13,28). This recommendation restricted the use of anti-EGFR mAbs to about 60% of all patients with *KRAS* wild type tumors (20). A meta-analysis of 45 clinical studies (29) concluded that *KRAS* mutations are predictive of survival, disease progression, and treatment failure in patients with advanced colorectal cancer treated with anti-EGFR antibodies. The benefits of anti-EGFR therapy were largely limited to *KRAS* wild type patients (29).

Unfortunately, not all patients with *KRAS* wild type status respond to anti-EGFR mAbs. The presence of *KRAS* mutations has low sensitivity and relatively high negative likelihood for determining non-responsiveness among the patients (30). One hypothesis to explain this could be the simultaneous or isolated presence of genetic aberrations of genes encoding the other downstream effectors of the EGFR mediated signal transduction pathway (31-34). This hypothesis was proven by the results of the following clinical studies which show that additional *RAS* mutation (*KRAS* exons 3 and 4 and *NRAS* exon 1, 2, 3, 4) analysis can help in further refining the treatment modalities.

Table 1 PRIME study, primary end points of (PFS and OS) efficacy results according to RAS mutation status

| Variable | FOLFOX4 + panitumumab (months) | FOLFOX4 (months) | P value |
|---|--------------------------------|------------------|---------|
| PFS | | | |
| Extended RAS wild | 10.1 | 7.9 | 0.004 |
| No KRAS exon 2 (12+13) mutation | 9.6 | 8.0 | 0.02 |
| Non KRAS exon 2 (12+13) mutation but other RAS mutation present | 7.3 | 8.0 | 0.040 |
| Extended RAS mutation | 7.3 | 8.7 | 0.001 |
| OS | | | |
| Extended RAS wild | 25.8 | 20.2 | 0.009 |
| No KRAS exon 2 (12+13) mutation | 23.8 | 19.4 | 0.03 |
| Non KRAS exon 2 (12+13) mutation | 17.1 | 17.8 | 0.01 |
| Extended RAS mutation | 15.3 | 18.7 | 0.001 |

Abbreviations: PFS, progression free survival; OS, overall survival.

Clinical evidence of presence of other genetic mutations in patients resistant to anti-EGFR therapy

In the era of personalized medicine various retrospective and prospective analyses are being conducted to search for more predictive biomarkers in the treatment protocol for various malignancies specially mCRC. As *KRAS* wild type status was not sufficient to ensure response to anti-EGFR mAbs, other predictive biomarkers (*KRAS*, *NRAS*, *BRAF* mutations, *PIK3CA* mutations and PTEN loss) from the signaling pathway were analyzed. Although the results are favorable for the predictive strength of some other genomic biomarkers, till now no recommendation has been made for extensive genotypic analysis before initiation of anti-EGFR antibody therapy (34-36).

A systematic review and meta-analysis by Yang *et al.* explored the association of *BRAF*, *PIK3CA* mutations and/or loss of PTEN expression with PFS, OS and objective response rate (ORR) among patients with *KRAS* wild type tumors treated with anti-EGFR mAbs were included. The authors concluded that *BRAF* mutations, *PIK3CA* mutations and loss of PTEN are promising biomarkers and can help in identifying the appropriate patients (37). In contrast, the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group (EWG) discouraged the testing of *BRAF*, *NRAS* or *PIK3CA*, and/or loss of expression of PTEN or AKT proteins for taking decisions regarding the administration of anti-EGFR antibody therapy among patients with mCRC (38).

These contradictory statements could not help in

establishing the status of other biomarkers in the algorithm of mCRC management. Later, the retrospective analysis of PRIME study by Douillard *et al.* initiated the concept of Extended RAS analysis (39). The prospective-retrospective analysis of PRIME study assessed the efficacy and safety of panitumumab plus FOLFOX4 (oxaliplatin, fluorouracil and leucovorin) as compared with FOLFOX4 alone, according to RAS (*KRAS* or *NRAS*) or *BRAF* mutation status. Of the study population, 48% patients had tumors with non mutated RAS (no *KRAS* or *NRAS* mutations in exons 2, 3, or 4) and rest had mutations in RAS (any *KRAS* or *NRAS* mutations in exon 2, 3, or 4). The administration of panitumumab-FOLFOX4 led to a significant improvement in PFS and OS (Table 1). In the subgroup of patients without RAS mutations, there was a significant improvement in PFS (P=0.004) and OS (P=0.04) with panitumumab-FOLFOX4, as compared with FOLFOX4 alone (39). Another subset (17%), consisting of those patients with wild type *KRAS* tumors, but with mutations in other RAS exons [non *KRAS* exon 2 codon (12 and 13) mutation, *KRAS* exon 3 (at codon 61) and exon 4 (at codons 117 and 146); *NRAS* exon 2 (at codons 12 and 13), exon 3 (at codon 61), and exon 4 (at codons 117 and 146); and *BRAF* exon 15 (at codon 600)], showed a non-significantly shorter PFS and OS in the panitumumab-FOLFOX4 group than in the FOLFOX4-alone group (Figure 1). These results were similar to those observed in the subgroup of patients with *KRAS* mutations in exon 2 in tumors (Table 1). Another important observation of the study was that the treatment effects were different between the subgroups of patients without RAS

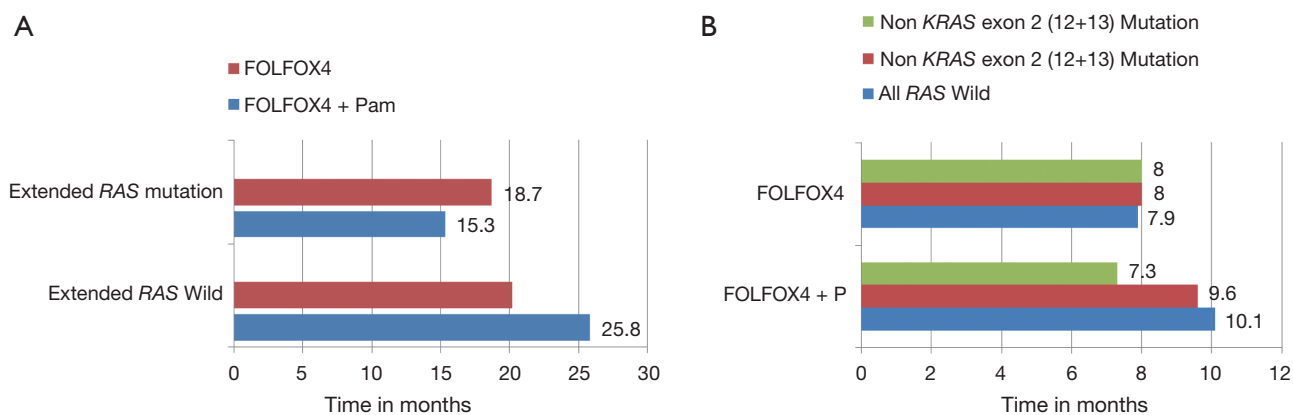


Figure 1 (A) PRIME study, in the wild *KRAS* exon 2 group FOLFOX + P improved progression free survival (PFS) when compared with FOLFOX only group (9.6 vs. 8 months respectively), the absolute magnitude of improvement of PFS is more pronounced when Extended *RAS* analysis is used to determine the *RAS* status with PFS 10.1 m in FOLFOX + P compared with 7.9 m in FOLFOX only group ($P=0.004$). The absolute improvement in PFS has increased from 1.6 months with wild *KRAS* exon 2 analysis to 2.2 months when Extended *KRAS* analysis is utilized; (B) PRIME study, clinically and statistically significant improvement in survival in FOLFOX and panitumumab in Extended *RAS* wild group when compared to FOLFOX alone, the presence of *RAS* mutation (any *KRAS* or *NRAS* mutations in exon 2, 3, or 4) in this population had detrimental effect on survival and did not drive any survival benefit from the addition of panitumumab in contrast to Extended *RAS* wild population where significant improvement in survival with FOLFOX and panitumumab comparing with FOLFOX only population (25.8 vs. 20.2 months respectively) ($P=0.009$).

mutations and those without *KRAS* mutations in exon 2 but with other *RAS* (*KRAS* or *NRAS* mutations in exons 2, 3, 4) mutations. This might suggest that *RAS* mutations, in addition to *KRAS* mutations in exon 2 codon (12 and 13), were negative predictive factors. The results suggest that presence of *RAS* mutations was a negative predictive factor. Further analysis showed that in the nonmutated *RAS* and nonmutated *BRAF* subgroup, panitumumab-FOLFOX4 was associated with a 1.6-month improvement in PFS and a 7.4-month improvement in OS, as compared with FOLFOX4 alone. Analysis of the prognostic effect of *BRAF* mutations showed that *BRAF* mutations were associated with reduced OS among patients without *KRAS* mutations in exon 2 and among those with *NRAS* mutations in exon 3. The safety profile for patients with *RAS* mutations was similar to that reported for patients with *KRAS* mutations in exon 2 (39).

Similarly, Soeda *et al.* while studying the response with cetuximab among irinotecan- and oxaliplatin-refractory Japanese patients with mCRC, found that the *KRAS*, *BRAF*, and *PIK3CA* wild type group had a better response rate and PFS than did the wild-type *KRAS* exon 2 subgroup (40). In the GERCOR efficacy, tolerance and translational molecular study, Andre *et al.* also studied *BRAF*, *NRAS* mutations and EGFR copy number in addition to the *KRAS* mutant status. Patients with *BRAF* mutations had a poorer prognosis and

lower response rates to anti-EGFR antibody therapy as compared to other groups. Evidence for rare *KRAS*, *NRAS* and *PIK3CA* mutations was poor because of small number of patients in these groups. The response was highly dependent on the mutant status of the patients and thus recommended an extended genotyping including rare *KRAS* and *NRAS* mutants (41).

The PEAK [panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6] study also assessed the treatment effect with an extended *RAS* analysis including exons 2, 3, 4 of both *KRAS* and *NRAS* among patients with previously untreated, unresectable, wild type *KRAS* exon 2 mCRC. Patients with wild type *RAS* tumors had better PFS ($P=0.029$) and median OS ($P=0.058$) with anti-EGFR therapy. PFS was similar and OS was better in the panitumumab group among the patients with wild type *KRAS* exon 2 tumors (42).

New evidence was presented at the American Society of Clinical Oncology 2014 and European Cancer Congress 2013 (25,43). Peeters *et al.* assessed the effect of second line treatment of panitumumab plus FOLFIRI (continuous infusion fluorouracil, oxaliplatin, and irinotecan) vs. FOLFIRI based on *RAS* mutation status in the population of the earlier study conducted in 2010. Mutations detected included *KRAS* exon 3, 4 and *NRAS* exons 2, 3, 4 in patients

Table 2 FIRE 3 study, primary end points of (PFS and OS) efficacy results according to *RAS* mutation status

| Variable | FOLFIRI + cetuximab (months) | FOLFIRI + bevacizumab (months) | P value |
|---|------------------------------|--------------------------------|---------|
| PFS | | | |
| <i>KRAS</i> exon 2 (12+13) wild | 10.0 | 10.3 | 0.55 |
| Extended <i>RAS</i> wild-type [Excluding all non <i>KRAS</i> exon 2 (12+13) mutation]* | 10.4 | 10.2 | 0.54 |
| OS | | | |
| <i>KRAS</i> exon 2 (12+13) wild | 28.7 | 25.0 | 0.017 |
| Extended <i>RAS</i> wild-type [Excluding all non <i>KRAS</i> exon 2 (12+13) mutation]* | 33.1 | 25.6 | 0.011 |

*exon 3 (codon 61), and exon 4 (codon 146), and *NRAS* exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146). Abbreviations: PFS, progression free survival; OS, overall survival.

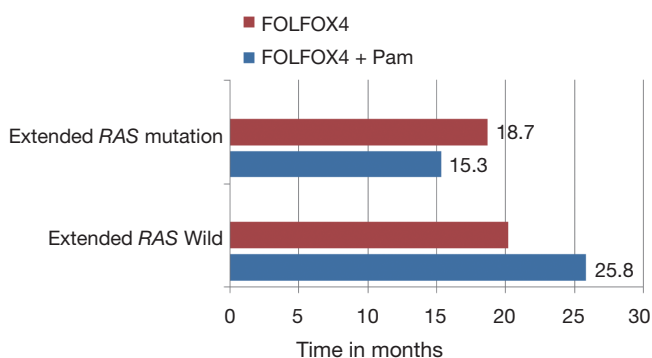


Figure 2 FIRE 3 study, clinically and statistically significant improvement in survival in FOLFIRI and cetuximab *KRAS* wild group in the preplanned analysis when compared to FOLFIRI and bevacizumab, the overall survival was more pronounced among Extended *RAS* wild type patients with the addition to cetuximab to FOLFIRI regimen as compared to addition of bevacizumab to FOLFIRI group.

with known *KRAS* wild type exon 2 mCRC. About 18% of the wild type *KRAS* patients had additional *RAS* mutations. The PFS and OS were better in the *RAS* wild type group as compared to *RAS* mutant group. Bokemeyer *et al.* studied *KRAS* exon 2 wild type patients from the OPUS study for 26 mutations (referred as *new RAS*) and additional *KRAS*, *NRAS* codons. New *RAS* mutations were present among 26% of patients. The patients from *RAS* wild type group showed significant improvement with addition of cetuximab to FOLFOX4 therapy. The distinctive observation of this study is that there was a trend towards worse outcome among patients with *RAS* mutation with the addition of cetuximab (26,44). Tejpar *et al.* (45) presented another set

of results from the OPUS study about the patients which were tested for *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4. The tumor status was available for 31% of patients and there was benefit among *RAS* wild type population with addition of cetuximab to FOLFOX4. There was a less favorable outcome and no benefit among *RAS* mutant population with addition of cetuximab (45). Ciardiello *et al.* studied the *new RAS* mutations among *KRAS* wild type exon 2 tumors from CRYSTAL study patients and *RAS* mutations were present in 15% of the patients. There was a significant benefit in all end points among *RAS* wild type patients with the addition of cetuximab to FOLFIRI regimen. Also, there was no benefit among the *RAS* mutant group with the addition of cetuximab (46). Stintzing *et al.* evaluated the effect of mutations in exon 3 (codon 61), and exon 4 (codon 146), and *NRAS* exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) on the ORR, PFS and OS among the *KRAS* (exon 2) codon 12/13 wild type patients. The ORR and OS were increased among *RAS* wild type patients with the addition to cetuximab to FOLFIRI regimen as compared to addition of bevacizumab to FOLFIRI regimen (Table 2, Figure 2) (47).

A recent systematic review and meta-analysis on alterations in *KRAS* exons 3 and 4, *NRAS*, *BRAF* and *PIK3CA* and *PTEN* and the outcome with anti-EGFR antibody therapy suggests that mutations in *KRAS* exons 3 and 4 and *NRAS* predict resistance to anti-EGFR mAbs. The ORR was significantly poor among those with *KRAS* mutation in exon 3 and 4 (odds ratio 0.26). The PFS was also significantly shorter due to mutations in *KRAS* exons 3 and 4 and *NRAS* (48). Sorich *et al.* have included all the above mentioned clinical trials assessing the role of

anti-EGFR mAbs for tumors harboring *RAS* mutations. They divided the patients from various RCTs into three subgroups. First group was the “*KRAS* exon 2” mutant group; second consisted of “*new RAS* mutant” (wild-type for *KRAS* exon 2, but with a *KRAS* mutation in exons 3 or 4 and/or a *NRAS* mutation in exons 2, 3 or 4) patients and third consisted of “Extended *RAS* wild type” patients. Tumors without any *RAS* mutations (either *KRAS* exon 2 or *new RAS* mutations) had significantly superior response [PFS ($P < 0.001$) and OS ($P = 0.008$)] with anti-EGFR mAb treatment as compared to tumors with any of the *new RAS* mutations. There was no PFS and OS benefit with anti-EGFR mAbs for tumors with any *RAS* mutations ($P > 0.05$) (49).

Discussion

Although in the initial years of use of anti-EGFR mAbs for mCRC, testing of *KRAS* exon 2 mutation helped in individualizing the treatment with anti-EGFR mAbs, yet, even after this analysis, a subset population of *KRAS* exon 2 wild type patients showed continues resistance to anti-EGFR agents. Since the isolation of *KRAS* mutant status as a lone negative predictor marker few years back to the present day scenario each and every step has been corroborated by evidence from clinical studies. The results of the above mentioned RCTs, systematic reviews and meta-analysis show that patients with tumors that are *KRAS* exon 2 wild-type (which includes both the “Extended *RAS* wild-type” and “*new RAS* mutant” subgroups) should not be considered to represent a single homogenous group for efficacy or resistance to anti-EGFR mAbs. The “Extended *RAS* wild-type” subgroup is distinct and has a significantly better response to anti-EGFR mAbs as compared to other patients. The response is indistinguishable among the *KRAS* exon 2 mutant patients and those with newly identified *RAS* mutations which include *KRAS* mutation in exons 3 or 4 and/or a *NRAS* mutation in exons 2, 3 or 4. Although the beneficial effects of anti-EGFR mAbs are explicit in the Extended *RAS* wild type group, results are still limited regarding the detrimental effects of anti-EGFR mAbs among *RAS* mutant groups (39,44). A broader analysis of mutant status can help in tailoring patient specific regimen and achieving maximum benefit. Thus based on the emerging benefit Extended *RAS* analysis, beyond *KRAS* exon 2, should be utilized in practice for predicting the benefit from the anti-EGFR mAbs among patients with mCRC.

Conclusions

The additional analysis of *KRAS* and *NRAS* genes as predictive markers can allow more accurate selection of patients who are more likely to benefit from anti-EGFR antibody therapy. Treatment with anti-EGFR mAbs should only be initiated after screening tumors for mutations in exon 2, 3 and 4 of both *KRAS* and *NRAS* genes. This will help in preventing unnecessary drug toxicity and associated expenses. Prior to the implantation of such recommendation there is a need to establish a standardized acceptable expanded *RAS* mutant status testing.

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