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 \textit{KRAS} gene before initiation of therapy with anti-EGFR mAbs among patients of mCRC (12,13).

\textbf{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\_\gamma pathway (15). \textit{RAS} is the most important superfamily of proteins, which includes mainly \textit{KRAS} and \textit{NRAS} proteins. \textit{KRAS} is a guanosine triphosphate cleaving enzyme (GTPase). The signaling through \textit{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 \textit{KRAS}, \textit{NRAS}, \textit{BRAF} or \textit{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 \textit{KRAS} gene, followed by mutations of \textit{NRAS}, \textit{PI3KCA} and \textit{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 \textit{BRAF} lead to uncontrolled \textit{BRAF} activation independent of EGFR and \textit{RAS} (17).

\textbf{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 \textit{KRAS} exon 2 wild type tumors only and not among those with \textit{KRAS} exon 2 mutant tumors (22,23). The median progression free survival (PFS) and overall survival (OS) significantly improved among the \textit{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 \textit{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 \textit{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 \textit{KRAS} is a negative predictive biomarker for therapy with anti-EGFR antibody therapy (11,22-27).

This led to the recommendation for routine \textit{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 \textit{KRAS} mutations. If \textit{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 \textit{KRAS} wild type tumors (20). A meta-analysis of 45 clinical studies (29) concluded that \textit{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 \textit{KRAS} wild type patients (29).

Unfortunately, not all patients with \textit{KRAS} wild type status respond to anti-EGFR mAbs. The presence of \textit{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 \textit{RAS} mutation (\textit{KRAS} exons 3 and 4 and \textit{NRAS} exon 1, 2, 3, 4) analysis can help in further refining the treatment modalities.
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=0.009) with panitumumab-FOLFOX4, as compared with 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>FOLFOX4 + panitumumab (months)</th>
<th>FOLFOX4 (months)</th>
<th>P value</th>
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<tr>
<td>PFS</td>
<td></td>
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</tr>
<tr>
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<td>8.0</td>
<td>0.040</td>
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<td>8.7</td>
<td>0.001</td>
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<tr>
<td>OS</td>
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<td></td>
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<td>20.2</td>
<td>0.009</td>
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<tr>
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<td>19.4</td>
<td>0.03</td>
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<tr>
<td>Extended RAS mutation</td>
<td>15.3</td>
<td>18.7</td>
<td>0.001</td>
</tr>
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Abbreviations: PFS, progression free survival; OS, overall survival.
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
with known \textit{KRAS} wild type exon 2 mCRC. About 18\% of the wild type \textit{KRAS} patients had additional \textit{RAS} mutations. The PFS and OS were better in the \textit{RAS} wild type group as compared to \textit{RAS} mutant group. Bokemeyer \textit{et al.} studied \textit{KRAS} exon 2 wild type patients from the OPUS study for 26 mutations (referred as \textit{new RAS}) and additional \textit{KRAS}, \textit{NRAS} codons. New \textit{RAS} mutations were present among 26\% of patients. The patients from \textit{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 \textit{RAS} mutation with the addition of cetuximab (26,44). Tejpar \textit{et al.} (45) presented another set of results from the OPUS study about the patients which were tested for \textit{KRAS} exons 3 and 4 and \textit{NRAS} exons 2, 3 and 4. The tumor status was available for 31\% of patients and there was benefit among \textit{RAS} wild type population with addition of cetuximab to FOLFOX4. There was a less favorable outcome and no benefit among \textit{RAS} mutant population with addition of cetuximab (45). Ciardiello \textit{et al.} studied the \textit{new RAS} mutations among \textit{KRAS} wild type exon 2 tumors from CRYSTAL study patients and \textit{RAS} mutations were present in 15\% of the patients. There was a significant benefit in all end points among \textit{RAS} wild type patients with the addition of cetuximab to FOLFIRI regimen. Also, there was no benefit among the \textit{RAS} mutant group with the addition of cetuximab (46). Stintzing \textit{et al.} evaluated the effect of mutations in exon 3 (codon 61), and exon 4 (codon 146), and \textit{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.

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<th>FOLFIRI + bevacizumab (months)</th>
<th>P value</th>
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</thead>
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<td></td>
</tr>
<tr>
<td>OS</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>\textit{KRAS} exon 2 (12+13) wild</td>
<td>28.7</td>
<td>25.0</td>
<td>0.017</td>
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<tr>
<td>Extended \textit{RAS} wild-type</td>
<td>33.1</td>
<td>25.6</td>
<td>0.011</td>
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<td>[Excluding all non \textit{KRAS} exon 2 (12+13) mutation]*</td>
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*exon 3 (codon 61), and exon 4 (codon 146), and \textit{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.

A recent systematic review and meta-analysis on alterations in \textit{KRAS} exons 3 and 4, \textit{NRAS}, \textit{BRAF} and \textit{PIK3CA} and PTEN and the outcome with anti-EGFR antibody therapy suggests that mutations in \textit{KRAS} exons 3 and 4 and \textit{NRAS} predict resistance to anti-EGFR mAbs. The ORR was significantly poor among those with \textit{KRAS} mutation in exon 3 and 4 (odds ratio 0.26). The PFS was also significantly shorter due to mutations in \textit{KRAS} exons 3 and 4 and \textit{NRAS} (48). Sorich \textit{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.

Acknowledgements

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References

8. US FDA. FDA Approves Erbitux for Colorectal Cancer.


