Background

Metastatic Colorectal Cancer (mCRC) is the third most common cancer and one of the leading causes of cancer-related death worldwide and accounting for 40% to 50% of newly diagnosed patients with high mortality rates. The 5-year overall survival (OS) is very low, which is 18 to 21 months even with the advancement of chemotherapeutic treatment. Two monoclonal antibodies (MoAbs), Cetuximab and Panitumumab, which target Epidermal Growth Factor Receptor (EGFR), have been approved more recently to treat mCRC. These two MoAbs target EGFR by binding to the extracellular domain and thus, leading to the inhibition of its downstream signaling. They have proven a modest clinical benefit in pretreated patients by the use of either, alone or in combination with conventional chemotherapy. It became clear from the beginning that not all the patients with mCRC benefit from these anti-EGFR MoAbs (1). Only 10% to 20% patients truly benefit from anti-EGFR MoAbs due to the high resistance against this therapy (2,3). Even the presence of wild-type KRAS does not guarantee the full benefit from anti-EGFR MoAbs therapy. In the absence of KRAS mutations, resistance to anti-EGFR MoAbs treatments may possibly be caused by the alterations in the downstream members of RAS-RAF-MAPK pathway?

Introduction

BRAF, one of the members of the three protein-serine/threonine kinases that are related to retroviral oncogenes, was discovered in 1988. Owing to prior DNA sequencing error, BRAF residue numbering changed in 2004. In the original version, residues after 32 were one number shorter than their actual position. BRAF is major downstream effectors of KRAS and is also considered an oncogene whose activating mutations appear in about 12-18% of human colorectal cancer (6). BRAF plays a role in the regulation of mitogen-activated protein/extracellular signal-regulated kinases MAP/ERKs signaling pathway, which controls the cellular division, differentiation and secretion. Mutations in this gene can lead to different diseases including CRC.

Factors involving in B-RAF mutations and impared signaling

The activation of BRAF oncogene, inactivation of mismatch repair genes by methylation of CpG islands, and microsatellite instability (MSI) have been reported to be involved in CRC development (7). B-RAF does not require additional negative charge during activation by additional enzyme modification, since its N-region contains an
activating serine site and the basal activity of BRAF is higher than its other RAF family members (8), that is why BRAF is more prone to mutations than CRAF and ARAF (9). Single amino acid substitutions can cause the activation of BRAF, but CRAF and ARAF require two mutations for their oncogenic activation, which is a very rare event to be seen (8). The most common BRAF mutation, which accounts for more than 90% of the cases of cancer involving this gene, is a glutamic acid for valine substitution at position 600 (V600E) (9). Continued expression of BRAF V600E is required for tumor growth and progression (10).

BRAF is a major contributor to many cancers. Somatic mutations in the BRAF gene have been detected in almost 50% malignant melanomas and many other cancers including CRC, ovarian and papillary thyroid carcinomas (11).

Of the oncogenic mutations in the BRAF gene, most are clustered in two regions of the kinase domain, which is responsible for maintaining the inactive catalytic conformation, the glycine rich loop and the activation segment. The proteins of BRAF oncogene with impaired kinase activity and the binding and activation of CRAF are required for ERK activation in vivo. The oncogenic BRAF proteins have been divided into three groups based on their enzymatic activity: (I) those with high enzymatic activity, they are 130-700 folds more active than the wild-type (WT) BRAF; (II) those with intermediate activity, which are 60 to 1.3 folds more active than WT BRAF; (III) those with impaired catalytic activity are 0.8 to 0.3 folds active as compared to WT BRAF (12). Activating mutations in BRAF oncogene have been reported in 10-15% CRC with the vast majority being a V600E hotspot mutation (13). V600E substitution is strongly associated with microsatellite instability (MSI+) phenotype, but is mutually exclusive with KRAS mutations (14). CIMP provides a unique opportunity to study the molecular mechanism that leads to epigenetic changes in CRC and how these changes can cause this disease (15). A strong association between CpG island methylator phenotype (CIMP) and the presence of an activated form of BRAF mutation (BRAFV600E) has been founded (16). It has also been demonstrated that sporadic microsatellite instability (MSI) occurs as a consequence of CIMP-associated MLH1 DNA hypermethylation (16,17). It has been reported that both BRAF mutations and CIMP are present in the earliest stages of colorectal neoplasia, where CIMP is present in apparently normal mucosa of patients predisposed to multiple serrated polyps (18) as well as BRAF mutations in aberrant crypt foci (19). BRAF mutations in tumors with MSI+ CIMP+ are 10 folds more frequent than tumors without these phenotypes (20), 70-80% BRAF mutation frequencies have been reported in sporadic MSI+, CIMP+ and MLH1-methylated CRC and polyps (21). The BRAF oncogene gene has been linked to MSI pathway in tumorigenesis (22).

BRAF mutation frequencies in MSI+ are much higher than MSI- tumors, and the higher frequencies have been seen in tumors showing methylation of the MLH1 promoter proximal region and in tumors with infiltrating lymphocytes (20). It has been reported in various studies that 100% of the carcinomas with BRAF mutations, methylation of hMLH1 occurred. Samowitz et al. have speculated about a fact, that MSI colorectal tumors that develop from hyperplastic polyps frequently show BRAF mutations and the methylator phenotype (CIMP), including the methylation of hMLH1 (23). According to Domingo et al., the inactivation of hMLH1 by methylation is reacted to the activation of BRAF, suggesting that specific modulation in the RAS/RAF system could occur depending on hMLH1 methylation status in CRC (24).

Koinuma K et al. reported an association between BRAF mutations and promoter methylation of the hMLH1 repair gene, where hMLH1 has been found to be altered in 80% of the cases of MSI sporadic CRC (25). BRAF mutations were reported to show prognostic significance in MSI - but not in MSI+ CRC (26). In various previous studies it has been reported that BRAF mutations in MSI-sporadic CRC are more frequently detected as compared with microsatellite -stable CRC (up to 50% vs. 12% respectively) (26).

During uncontrolled division in tumor cells, their demand for nutrients and oxygen increases, and to adapt to hypoxic environment, cells switch to anaerobic glycolysis and induction of survival factors and angiogenic growth factors such as; vascular endothelial growth factor (VEGF) (27). In hypoxia, Hypoxia-inducible factors (HIFs) are thought to play a major role in controlling the transcriptional responses (28). Mutated BRAF induces and regulates both Hypoxia-inducible Factor-1α (HIF-1α) and Hypoxia inducible Factor -2α (HIF-2α) in hypoxia (29). KRAS induces only HIF-1α. HIF-1α is thought to promote the growth of colon cancer cells, whereas; HIF-2α may restrain growth. The differential effects of KARS and BRAF mutations on the HIFs presents the unique interaction between the oncogenes and the tumor microenvironment, which may provide the phenotypic differences in mutant BRAF and KRAS CRC (29).

**MoAbs action with non-BRAF mutated and BRAF mutated cells**

Two monoclonal anti-bodies (MoAbs), Cetuximab and Panitumumab, which target the EGFR have been entered in clinical practice to treat mCRC, both these molecules bind to the EGFR external domain, leading to inhibition of its downstream signaling pathways (Figure 1). These include
the RAS-RAF-MAPK axis, which is mainly involved in cell proliferation, and the PI3K-PTEN-AKT pathway, which is involved in cell survival and motility (30).

The anti-EGFR monoclonal antibody, Cetuximab, has demonstrated clinical benefits in, and is widely used to treat, mCRC (Figure 1) (31). Notion has been acknowledged by European Medicine Agency (EMEA), which approved the use of Panitumumab or Cetuximab only in mCRC patients whose tumors display wt-KRAS (32). American Society of Clinical Oncology recommended that only those mCRC patients with wild-type KRAS be considered candidates to receive anti-EGFR therapy. The efficacy of anti-EGFR monoclonal antibodies in 60-70% of mCRC patients with wt-KRAS tumors is still limited, with response rates between 10% and 40% (33). There is a need for additional biomarkers for these patients. Interestingly the expression of the EGFR protein has not been strongly associated with clinical response to Cetuximab in CRC, although, there is limited evidence that amplification of the EGFR gene relates to objective response and other indices of clinical benefits. The relation between the increase of the EGFR gene dosage and response to Cetuximab or Panitumumab is not strong enough to allow the clinical use of this biomarker for the predictive selection of patients (34).

As proven, BRAF is the principal effectors of KRAS (35) and its oncogenic V600E mutation is mutually exclusive with KRAS mutations in CRCs (36). It has been demonstrated that the V600E mutation can also preclude responsiveness to Panitumumab or Cetuximab in mCRC patients and cellular models of CRC also, mutations in BRAF have shown impaired responsiveness to Panitumumab or Cetuximab in patients with mCRC (Figure 2A) (4). Of note, KRAS and BRAF mutations are known to be mutually exclusive in colorectal cancers (36). Patients who have mutated BRAF don’t respond to MoAbs therapy even if they present wt-KRAS, which shows that wt-BRAF is required to respond to MoAbs therapy to treat mCRC (4). Therefore, mutated BRAF tumors (approximately 10%) add algebraically to those carrying KRAS mutations (35-45%), thus further empowering the selection of patients eligible for Cetuximab/Panitumumab treatment. Of note, when considered together, the two biomarkers can identify up to 55% non-responders (4).

**BRAF inhibitors**

As there are no drugs currently available for the specific and

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**Figure 1** A. Normal binding of ligand to EGFR and activation of downstream signaling transduction cascade leading to DNA synthesis, cellular proliferation and migration etc; B. Binding of anti-EGFR drug e.g., cetuximab or panitumumab to EGFR which inhibits ligand binding to EGFR and inhibits receptor’s activation and resulting downstream signaling cascade of RAS-MAPK pathway’s activation inhibition, shown as red line, leads to the inhibition of cellular proliferation, migration, and DNA synthesis etc.
direct inhibition of KRAS, but there are number of agents that are designed to inhibit the kinase activity of BRAF, which is either already clinically approved or is progressing through the pipeline of phase I and II studies (37).

The pharmacological inhibition of the MAPK signaling cascade in cancer cells carrying constitutively active KRAS and BRAF mutants has been shown to improve anti-EGFR treatment with MoAbs. In this regard, it has been reported that treatment with the BRAF inhibitor, Sorafenib, can restore sensitivity to Cetuximab and Panitumumab of CRC cells carrying the V600E allele (38).

So, the concomitant treatment of patients with mCRC bearing BRAF-mutated tumors, with Cetuximab/Panitumumab in combination with a BRAF inhibitor, is possible and supported by a strong rationale. MoAbs activity can be restored in BRAF mutated patients by introducing BRAF inhibitor along with MoAbs therapy (Figure 2B). Recently another study has reported the preclinical characterization of Vermurafenib (RG7202;PLX4032;RO5185426), which is a first-class specific small molecule BRAFV600E inhibitor in BRAF-mutated CRC cell lines and tumor xenograft models. In the same study Vermurafenib showed the dose dependent inhibition of ERK and MEK phosphorylation, which caused the inhibition of tumor growth in BRAFV600E, bearing xenograft models and arresting of cell proliferation in BRAFV600E expressing cell lines. This shows that combination of Vermurafenib with MoAbs therapy could enhance the clinical anti tumor efficacy in CRC harboring the BRAFV600E mutation (Figure 2B) (39). It has been shown that the multikinase inhibitor, Sorafenib, might restore sensitivity to EGFR inhibitors in BRAF mutated CRC cell lines, and combining of more selective BRAF inhibitors [e.g., PLX-4032 and XL-281 can also restore the sensitivity EGFR-targeted antibodies towards BRAF mutation (4)]. The first generation of RAF inhibitors, including sorafenib, were notable for their lack of specificity and potency for RAF and these agents have shown limited efficacy in tumors with a high incidence of BRAF mutation, such as, melanoma. Novel inhibitors of the pathway with greater selectivity for BRAF and MEK are now in Phase 1 and 2 clinical trials with promising early results.

To maximize the likelihood of success with these agents, clinical trials enriched with patients whose tumors possess

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Figure 2 A. Inactivation of EGFR by anti-EGFR drugs does not inhibit the activation of RAS-MAPK pathway due to BRAF oncogene mutation, shown in red, which causes uncontrolled cellular proliferation, migration, and survival etc; B. Combination of cetuximab/panitumumab and BRAF inhibitor i.e., Vermurafenib PLX4030 inhibit the downstream signaling cascade, oncogenic proliferation and survival in BRAF mutated cells.
BRAF and RAS mutations have been proposed (40). It has also been reported that AZ628, a selective and potent investigational small molecule RAF kinase inhibitor, is remarkably effective at inhibiting the growth of a specific subset of human cancer cell lines derived from melanomas, thyroid cancers, and colorectal cancers that harbor the BRAF V600 mutation (41).

**Resistance to BRAF inhibitors**

Clinical responses to target anticancer therapeutics are frequently confounded by de novo or acquired resistance (42). In chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GIST) and non-small cell lung cancers (NSCLCs), acquired resistance to kinase inhibitors is frequently associated with either secondary kinase domain mutations, amplification of the gene encoding the target kinase, or mutational activation of genes encoding components of alternative survival pathways (42-46). It has been shown that elevated levels of CRAF cause the acquired resistance to BRAF inhibition in the melanomas (47). It has also been shown that MAP3K8 (COT/TPL2), which is a MAPK pathway agonist, drives resistance to RAF inhibition in cell lines containing RAFV600E mutation (48).

**Overcoming BRAF inhibitors’ resistance**

As various targeted kinase inhibitors have demonstrated both pre-clinical and clinical activity, the application of these agents to large patient population has clearly demonstrated that while initial clinical responses can be dramatic, rapid acquisition drug resistance is a major limitation to the over therapeutic efficacy of these drugs. Therefore, one of the major challenges associated with the border use of these inhibitors is the elucidation of drug resistance mechanisms and the development of strategies to overcome or prevent resistance.

Identification of resistance mechanisms in a manner that elucidate alternative “druggable” targets may inform effective long-term treatment strategies (49). Each of these identified resistances in CLM, GIST and NSCLC, has been successfully modeled in cell culture using appropriate drug-treated cancer cell lines, indicating that such cell culture modeling can provide an effective system for identifying mechanism of acquired drug resistance that are likely to arise clinically (46,50,51). This is important because the development of strategies to overcome drug resistance, which will generally requires considerable time, first requires the identification of relevant resistance mechanisms. Therefore, the ability to anticipate clinical mechanisms of acquired resistance to targeted kinase inhibitors is likely to greatly accelerate the development of strategies to overcome drug resistance (52), and to reduce the current temporal gap between initial clinical successes and subsequent disease progression in the absence of available secondary treatment options. Anticipating the potential mechanisms of acquired resistance that could develop to the RAF inhibitors during the course of treatment can overcome this problem, as drug resistant clones from human melanoma-derived cell line harboring the V600E activating mutation that showed sensitivity to AZ628, a selective RAF kinase inhibitor. In the subset of these clones, significantly increased expression of the BRAF-related CRAF protein appeared to account for the acquisition of resistance to AZ628. But the resistant clones, which have shifted their dependency from BRAF to CRAF, acquired substantial sensitivity to the HSP90 inhibitors, Geldanamycin, which promotes the degradation of CRAF, thereby revealing a potential therapeutic strategy to overcome this resistance mechanism (47).

**BRAF mutation analysis in CRC**

As in July 2009, the Food and Drug Administration (FDA) approved labeling changes to two EGFR antagonists, Cetuximab and Panitumumab, stating that these agents are not recommended for the treatment of CRC harboring KRAS mutations. Thus, determination of KRAS mutation status in these tumors is critical when evaluating a patient for anti-EGFR therapy.

The American Society of Clinical Oncology (ASCO) has further recommended that all patients with metastatic colorectal cancer, for whom EGFR antagonists are being considered, should be specifically tested for KRAS mutational status at codons 12 and 13. Current guidelines in the US state, that patients with metastatic CRC being considered for EGFR-targeted therapies should be tested for KRAS and BRAFT mutations (53)

Data from the CRYSTAL trial suggest that BRAF mutations are also indicative of poor prognosis and the National Comprehensive Cancer Network (NCCN), Colon Cancer Guideline Update 2010 states that testing for mutations in BRAFT should occur when KRAS testing indicates KRAS wild type, to avoid exposing patients to ineffective drugs, exposure to unnecessary drug toxicities, and expedite the use of the best available alternative therapy (54). High-resolution melting (HRM) is a recently developed technique that shows great potential for scanning germline and somatic mutations (55).

Also, another recent study described HRM assay for mutation detection in EGFR exons 19-21, KRAS codon 12/13 and BRAF V600 using formalin-fixed paraffin embedded samples, which proved HRM as a rapid and
sensitive method for moderate-throughput cost-effective screening of oncogene mutations in clinical samples (56).

BRAF mutations are now increasingly being investigated in metastatic colorectal cancer. KRAS mutation analysis may be considered medically necessary to predict no response to anti-EGFR monoclonal antibodies Cetuximab and Panitumumab in the treatment of metastatic, unrespectable, or advanced colorectal cancer.

BRAF mutation analysis is considered investigational for all indications, including, but not limited to, predicting no response to anti-EGFR monoclonal antibodies Cetuximab and Panitumumab in the treatment of metastatic, unrespectable, or advanced colorectal cancer.

KRAS and BRAF mutation analyses using PCR methodology are commercially available as laboratory-developed tests. Such tests are regulated under the Clinical Laboratory Improvement Amendments (CLIA). Premarket approval from the U.S. Food and Drug Administration (FDA) is not required when the assay is performed in a laboratory that is licensed by CLIA for high-complexity testing.

**Factors affecting the efficacy of MoAbs Cetuximab In CRC patients other than BRAF, KRAS mutations**

Mutated BRAF tumors (approximately 10%) add algebraically to those carrying KRAS mutations (35% to 45%), thus further empowering the selection of patients eligible for Cetuximab/Panitumumab treatment. When considered together, the two markers can identify up to 55% of non responders (4). Results from other groups recently reported at the 2009 annual meeting of the American Association of Cancer Research and the American Society of Clinical Oncology confirmed these data (57).

In addition to KRAS and BRAF, the EGF receptor also activates the PI3k signaling pathway. This signaling pathway can be oncogenically deregulated either by activating mutations in the PIK3CA p110 subunit or by inactivation (often by epigenetic mechanisms) of the PTEN phosphatase. The role of deregulated PIK3CA/PTEN signaling on the response to Cetuximab and Panitumumab has therefore been investigated. As in one study, it is indicated that when expression of PTEN and mutations of KRAS, BRAF and PIK3CA concomitantly ascertained up to 70% of patients with mCRC unlikely to respond to anti-EGFR therapies, can be identified (58). A gross analysis of current data regarding the impact of PIK3CA mutations and PTEN loss on response is conflicting (59-63). From the published work, it seems that PIK3CA mutations are in fact associated with the resistance, although, this correlation is nowhere close to that observed for KRAS or BRAF. However, most of the authors agree that PTEN inactivation is a negative predictor of response (59,64).

As KRAS and BRAF mutations are exclusive, but the mutations of PIK3CA or inactivation of PTEN can coexist [i.e., they can occur in the same tumor containing KRAS/ BRAF mutations (3),], which makes it difficult to find the individual contribution of PIK3CA mutations and PTEN inactivation to the resistance against MoAbs therapy other than KRAS and BRAF mutations. It has also been shown that PIK3CA mutations located in exon 9 and 20 hotspots exert different biochemical and oncologic properties and are differently activated by KRAS (65). So, it is convincible that both PIK3CA mutations and PTEN inactivation have a little contribution of resistance against Cetuximab and Panitumumab therapy due to co-occurrence of PTEN expression and PIK3CA mutations with KRAS and BRAF mutations and different oncogenic properties of different PIK3CA mutations, so for definite conclusions more research work and analyzing of large cohorts of patients are needed to become useful to further analyze the eligible patients to treat with MoAbs therapy. However, these two markers are not yet ready to use clinically. Other possibilities can be the occurrence of alterations in other key elements of the EGFR-dependent signal cascade (e.g., AKT1 or MEK- MAPK), as in preclinical studies, inhibition of the MEK kinase effectively and specifically inhibits the growth of human tumor cell lines harboring activating BRAF mutation (66) and genetic alternation in tyrosine kinase receptors other than EGFR, providing an alternate pathway of survival and/or proliferation.

The Relationship between the increase EGFR gene dosage and response to Cetuximab or Panitumumab is not strong enough to allow the clinical use of this biomarker for the predictive selection of patients (34).

Secondary resistance to MoAbs therapies in mCRC patients is another cause of ineffectiveness, therefore, it is important to identify the possible mechanism causing secondary resistance. As has been mentioned in a clinical data, the response is transient, even in the KRAS and BRAF wild type tumors, and only last for 1 to 1.5 years (67).

The somatic knocking-out or knocking-in of individual alleles in normal or neoplastic cells is a new generation of cell tumor progression models, which has been developed recently. Generation of paired cell lines which closely recapitulate the occurrence of cancer mutations in individual patients as a result of targeting the endogenous loci for mutation or correction (68,69). It has been shown that the growth of human tumor cell lines harboring activating BRAF mutations can be inhibited by effective and
specific inhibition of MEK kinase (66).

**Role of ethnicity, gender and smoking in BRAF mutated mCRC**

The link of BRAF and KRAS mutations with ethnicity has been reported. In Chinese and Caucasians BRAF mutations were reported to be associated with advanced disease stages and worse survival of papillary thyroid microcarcinoma (70,71), but not in Japanese (54). A study from Australia showed that people of southern Europe origin had a lower risk of BRAF mutation then those of Anglo-Celtic origin (72). BRAF mutations were detected in about 45% of the high microsatellite instability (MSH-H) tumors and in about 10% of the microsatellite stable (MSS) tumors in Caucasians (73). In African Americans, distinct BRAF mutation has been reported, with 23% in MSI tumors and 0% in non-MSI tumors (74). These findings show the relation and importance of investigation of BRAF mutations with different ethnic groups.

In colorectal cancers, BRAF and PIK3CA (but not KRAS, APC, or TP53) mutations display a gender bias at higher frequencies in females (75,76). This suggests that tumors with BRAF somatic mutations arise from a different pathway in women. As one study has reported that exposure to estrogen in women protects against MSI, whereas, the lack of estrogen in aged females increases the risk of instability (77). Use of Hormone Replacement Therapy (HRT) significantly reduces the risk of colon cancer in postmenopausal females (78). This shows that the lack of female hormones contributes in the development of various cancers including colon cancer, which suggests that it could be hypothesized that female patients with mCRC might be less likely to benefit from treatment with EGFR-targeted MoAbs. However, available clinical data do not support this hypothesis (79,80).

Smoking is also associated with mCRC caused by BRAF mutations but it is not as strongly associated as gender, though females are twice likely to smoke and have a tumor with BRAF mutation, but it is not strongly associated with smoking, as men who smoke are at higher risk of mCRC with BRAF mutations (81).

**Conclusions**

In addition to KRAS analysis, BRAF analysis should be done to rule out the BRAF mutations, especially in the developing countries like China, where BRAF testing is not common (other than few metropolitan areas), to avoid the unnecessary cytotoxicity, for selecting patients who would respond to the therapies and as the shocking costs of these targeted therapies, so the selection of patients is the key role to their economic sustainability. And besides using Anti-EGFR MoAbs, other alternative therapies should also be considered. As current data suggests that evaluation of not only KRAS or BRAF but also PIK3CA/PTEN alterations could be useful for selecting patients with mCRC who are unlikely to respond to anti Anti-EGFR-MoAbs. Genetic manipulation techniques can be applied to cellular models, one can envisage developing in vitro tools to prospectively find new sensitivity resistance biomarkers that can then be confirmed in patients and even be used to screen for rationale drug combinations to reverse resistance. To restore the sensitivity of MoAbs, they could be administered along with BRAF inhibitors and at the same time new ways should be found out in order to reduce the resistance to the BRAF inhibitors, further understanding of the molecular mechanisms to discover new alternative therapies and tests for non-responding patients would be helpful.

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