Commentary

Impact of genomic heterogeneity associated with acquired anti-EGFR resistance in colorectal cancers

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Cetuximab and panitumumab are anti-EGFR monoclonal antibodies approved by the Food and Drug Administration of the Unites States for treatment of patients with metastatic colorectal cancers, but the response rate is only around 10% in unselected patient populations (1). Despite initial response to cetuximab and panitumumab, almost all patients develop resistance within several months of anti-EGFR-therapy. Mechanisms for acquired resistance to targeted therapeutics with small molecule kinase inhibitors have been extensively studied, because new inhibitors can be designed to overcome the resistance mutations which commonly happen in the kinase domain of the targeted kinase (2). For example, the most common resistance mutation, *EGFR* p.T790M, is detected in 50–60% or more of lung cancer patients who progressed during treatment with first-generation or second-generation EGFR tyrosine kinase inhibitors (3). Detection of p.T790M has become a common clinical practice to select patients with non-small cell lung cancers for third-generation tyrosine kinase inhibitors.

One of the major obstacles for studying acquired anti-EGFR resistance in colorectal cancers is the limited access to resistant tumors due to lack of second-line targeted therapy and risk of biopsy. Over the last few years, blood-based analysis of circulating cell-free tumor DNA (ctDNA) (or liquid biopsy) has provided a non-invasive approach to reveal the diverse and dynamic genetic landscape of acquired resistance for anti-EGFR therapy in colorectal cancers (4-8). In contrast to small molecule kinase inhibitors, mechanisms for acquired resistance to anti-EGFR monoclonal antibodies are significantly overlapped with those for primary resistance, though with few exceptions (9). These include mutations in the *KRAS*, *NRAS*, and *BRAF* genes to bypass the mitogen-activated protein kinase pathway as well as amplification of *MET* and *EBRR2* genes to activate parallel pathways. One of the exceptions is the p.S492R mutation within the EGFR extracellular domain, which has not been reported in colorectal cancers prior to anti-EGFR therapy. Mutations involving codons 464 and 465within the extracellular domain as well as codons 714 and 794 within the kinase domain have also been reported as acquired resistance mechanisms (6,7). In addition, mutations of codon 61 in either *KRAS* or *NRAS* gene, instead of codon 12 and 13 in the *KRAS* gene, are more common for acquired resistance than for primary resistance. Recently, the landscape has been expanded to include mutations in *ERBB2*, *EGFR* (p.G465R and p.G465E), *FGFR1*, *PDGFRA* and *MAP2K1* genes as potential mechanisms for primary anti-EGFR resistance (10). Understanding the whole picture of primary or acquired genetic alterations may pave the road to overcome anti-EGFR resistance.

RAS mutations account for most genetic alterations seen in colorectal cancers with primary or acquired resistance to anti-EGFR therapy. Resistant tumors with "acquired" *RAS* mutations may arise from a small subpopulation pre-existing within the original tumor or a newly emerged subpopulation as a consequence of continued mutagenesis

over treatment courses. In the stepwise genetic alteration model associated with colorectal tumorigenesis, *RAS* mutations occur during the progression from small adenoma to large adenoma (11). Therefore, *KRAS* mutations are supposed to be present in all invasive components of *KRAS*-mutated colorectal cancers. This model, however, does not exclude the possibility that *RAS* mutations may occur after establishment of the founder cells for invasive carcinoma. "Acquired" *KRAS* mutations could be detected in plasma ctDNA 5 to 6 months before radiographic evidence of disease progression (4). A mathematical model suggested that low-frequency subclones harboring these *KRAS* mutations were present in the tumor before anti-EGFR treatment (4). In our retrospective quality assessment of a next generation sequencing assay conducted in colorectal cancers, lower than expected mutant allele frequency suggests that the mutant clone is present in a subpopulation, which was confirmed by analysis of different subareas (12). These findings support genetic heterogeneity in colorectal cancers.

Heterogeneity of genomic alterations is common in human cancers (13,14). After the founder clone has been established by collaborative oncogenic effects of the trunk (or initiating) driver mutations, subclones emerge as a consequence of evolutionary selection by continuous endogenous changes of the cancer (such as branch driver mutations) in combination with diverse exogenous conditions of the environment (such as therapeutic intervention by targeted therapy). Massively parallel sequencing or next generation sequencing technology has led to a revolution in genome research and precision cancer medicine, including the discovery of driver mutations suitable for targeted therapy as well as molecular heterogeneity pivotal for resistance to targeted therapy. Currently, most targeted therapeutic agents are designed to block oncogenic effects of initiating driver mutations, but tumor heterogeneity may pose a major challenge to acquired resistance.

A well-known example is non-small cell lung cancer with coexisting acquired resistance mutations and/or transformation of small cell carcinoma, suggesting multiple resistant clones exist (15), though a clone with multiple resistance mechanisms is also possible. We and others have observed that *EGFR* p.T790M mutation was present only in the adenocarcinoma component but not in the small cell carcinoma component in patients with both resistant adenocarcinoma and transformation of small cell carcinoma. This reciprocal relationship between p.T790M mutation and small cell carcinoma transformation supports genomic heterogeneity associated with acquired resistance in lung cancers (16). In colorectal cancers, detection of multiple *RAS* mutations in plasma ctDNA also suggests the presence of multiple resistant subclones (4,6,8).

In a recent article described by Russo *et al.*, tumor heterogeneity associated with acquired resistance to anti-EGFR therapy was confirmed in a patient with colorectal cancer (17). More importantly, the authors demonstrated how genomic heterogeneity drives lesion-specific response to subsequent targeted therapeutics. After treatment with cetuximab for 15 months, a liver biopsy of a metastatic lesion revealed a novel *MAP2K1* (*MEK1*) p.K57T mutation. A combinatory panitumumab and trametinib (MEK inhibitor) treatment led to regression of this metastatic lesion, while another liver metastatic lesion progressed. Retrospective analysis of plasma ctDNA prior to panitumumab and trametinib treatment revealed not only *MAP2K1* p.K57T mutation but also an additional *KRAS* p.Q61H mutation, which was also present in the progressed liver lesion. Serial follow-up of plasma ctDNA showed significant decline of the *MAP2K1* mutant level, but marked elevation of the *KRAS* mutant level during the treatment course. The authors also concluded that single-lesion tissue biopsy, the current diagnostic standard, may fail to detect clinically relevant resistant mutations due to genomic heterogeneity associated with acquired resistance. Analysis of plasma ctDNA can potentially provide more wide-angle mutational profiling than single-lesion biopsy and also allow real-time monitoring of dynamic changes of tumor load.

One potential strategy that has been proposed to overcome genomic heterogeneity associated with acquired resistance is to design targeted therapy aimed at blocking the convergent pathway of different resistance mutations or the intersected node of multiple pathways (8,9). One or more acquired mutations involving genes in the mitogen-activated protein kinase pathway were detected in 96% of anti-EGFR resistant patients (6), illustrating genetic heterogeneity, but with biochemical convergence to maintain activation of downstream MEK and ERK. Preclinical studies have also shown that anti-EGFR resistance can be reversed by concomitant inhibition of EGFR and MEK (8). Another potential strategy is to preclude "acquired" resistance by combining cetuximab or panitumumab with other kinase inhibitors targeting a specific resistance mutation present in a minor subpopulation of the tumors at the beginning of targeted therapy. Preclinical studies have shown the efficacy of combined therapy with anti-EGFR and MEK or ERK

inhibitor for *MAP2K1* mutations, anti-EGFR and imatinib for *PDGFRA* mutations, etc. (8,10). However, identification of a minor resistant subpopulation present before anti-EGFR therapy could be challenging.

From the diagnostic point of view, mutational profiling of plasma ctDNA by next generation sequencing assays offers the most comprehensive approach for detection of resistance mutations and avoids the spatial sampling bias of biopsied tissue specimens. We and others have shown that next generation sequencing is a robust tool for comprehensive mutational profiling in clinical laboratories. It demonstrates high analytic sensitivity (2–5% mutation allele without special design) and broad reportable ranges (from a panel of genes to whole exome sequencing) to detect initiating driver mutations for targeted therapy (12,18). Although the analytic sensitivity of next generation sequencing assays may be sufficient to detect acquired resistance mutations in plasma ctDNA after tumors progressed, an ultra-sensitive assay, such as digital droplet PCR, may be needed to identify primary resistance mutations present in a small subpopulation before targeted therapy. Digital droplet PCR assays have been clinically validated for detection of acquired *EGFR*. p.T790M mutation in plasma ctDNA. It's clinical application in colorectal cancers without a dominant acquired resistance mutation is limited due to lack of capability for comprehensive mutational profiling when the amount of ctDNA is inadequate.

In a clinical diagnostic setting, the analytic sensitivity of an assay is dictated not only by the capability of the assay to detect the targeted mutants, but also by the background noise of the assay (analytic specificity) as well as the total DNA input to prevent the false positive and/or false negative results (19), especially when an ultrasensitive assay is implemented. In next generation sequencing assays, sufficient depths of coverage for the claimed analytic sensitivity are also necessary (19). In addition, while comparison of results from paired tissue biopsy and liquid biopsy has shown appropriate clinical sensitivity of detecting *EGFR* p.T790M or *RAS* mutants in ctDNA (6,7,20), more studies are needed to validate the clinical specificity. Is detection of a relatively common mutation in human neoplasms, such *KRAS* or *BRAF* mutation, in plasma ctDNA sufficient to conclude that the mutant is present in the resistant tumor? What is the incidence of *KRAS* mutants detectable in plasma ctDNA from "normal" individuals or individuals with colorectal adenomas? Such and more questions remain to be answered.

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