

Resistance to EGFR targeted antibodies - expansion of clones present from the start of treatment. The more things change, the more they stay the same (Plus ça change, plus ça ne change pas!)

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The management of metastatic colorectal cancer (mCRC) has evolved with the development of targeted therapies. Two monoclonal antibodies, panitumumab and cetuximab, directed against the epidermal growth factor receptor (EGFR), have efficacy either in combination with chemotherapy or alone in the chemotherapy-refractory setting (1-3). They function by blocking ligand binding to the extracellular domain of EGFR, preventing downstream signalling. However, not all patients with colorectal cancer benefit from treatment with these agents (4,5). The clinical observation of variations in response between patients is now understood at a molecular level to be due to aberrant activation of downstream ERK 1/2 signalling. Constitutive activation of this pathway, bypassing the inhibited EGFR, can result from mutations in KRAS, BRAF and NRAS, which result in *de novo* clinical resistance to anti-EGFR antibodies (6). The clinical benefit and utility of these agents are restricted to patients with tumours without KRAS mutations (wild type) and consequently tumour samples from patients with mCRC are routinely tested for KRAS mutations before consideration of anti-EGFR therapy (4,5). While BRAF and NRAS mutations occur less frequently and are not currently used to select patients for therapy, they are also likely to be important predictive factors for response to anti-EGFR therapy.

However, even molecularly enriched subjects who initially respond to anti-EGFR antibodies ultimately acquire resistance to these agents, usually within 5-11 months. Unlike the case with small molecule tyrosine kinase inhibitors with which mutations mediating acquired resistance have been demonstrated, it has been previously

uncertain as to the mechanisms leading to resistance to EGFR targeting monoclonal antibodies. Improved understanding of the mechanisms of acquired resistance is essential for the development of rationally designed interventions to overcome or prevent the emergence of resistance. Some studies have supported the hypotheses of the acquisition of new mutations and/or activation of alternate signalling pathways as underlying the development of acquired resistance. However, in a recent issue of *Nature*, two articles provide support to the existing clone hypothesis - that the emergence of resistance is inevitable due to the pre-existing presence of low, unrecognised levels of resistant cells that expand during treatment to become the dominant tumour cell type (7,8). Diaz Jr *et al.* identified accumulating KRAS mutations in patients developing resistance to panitumumab, with mathematical modelling suggesting these mutations must have pre-existed in tumour cells prior to therapy, with the resistant subclones expanding following successful treatment of sensitive tumour cells. Furthermore, the authors add to a growing body of evidence that mutations can be detected through minimally invasive measures using a form of “liquid biopsy” (7), utilising circulating DNA fragments identified in serial plasma samples. Prior to reviewing these results in detail, it is worthwhile examining the current understanding of resistance mechanisms to anti-EGFR antibodies.

Mechanisms of acquired drug resistance can be broadly divided into: secondary mutations in the drug target itself that block the binding of, or the inhibitory activity of the drug; and activation of alternative signalling pathways that bypass the original, inhibited target. *In vitro* models of

acquired resistance have been developed through prolonged exposure of cetuximab-sensitive human colorectal cancer cell lines to cetuximab. The resistant cells generated have been found to contain a secondary EGFR mutation (S492R) in the extracellular domain that impairs binding of cetuximab. This mutation has also been identified in the tumours of patients with clinical resistance to cetuximab (9). Additionally, activation of ERBB2 signalling has been identified as a bypass mechanism of resistance through amplification of the ERBB2 receptor and increased levels of the EGFR ligand heregulin (10).

In addition to these mechanisms of acquired resistance, Diaz Jr *et al.* hypothesised that tumours classified as KRAS wild-type might harbour low levels of KRAS-mutant cells even prior to the commencement of targeted therapy. To test their hypothesis, the authors retrospectively examined a cohort of 28 patients with chemotherapy-refractory mCRC receiving single-agent therapy with panitumumab. Based on archival tumour tissue, 4 patients had KRAS mutations while the remainder were KRAS wild-type (7).

To monitor for the emergence of common KRAS mutations during treatment, Diaz Jr *et al.* retrospectively examined circulating tumour-derived free DNA (ctDNAs) in longitudinal serum samples from the 28 patients through the use of a digital ligation assay, with the results quantified by a PCR assay. They selected this method in order to overcome issues of limited post-treatment tissue availability and potential sampling bias due to tumour heterogeneity (7). Evaluation of ctDNAs has gathered considerable interest across a range of malignancies as a minimally invasive technique with the potential to provide real-time assessment of the genetic markers of a tumour and have possible predictive and prognostic roles. It also has the potential to overcome a number of issues associated with the current, most commonly utilised technique of assessment of KRAS mutations from DNA isolated from formalin-fixed, paraffin-embedded biopsy or resection samples. This current method can be hampered by insufficient DNA of adequate quality for biomarker analysis, limited access to samples at times of disease progression and sampling bias due to tumour heterogeneity. While results to date have yet to change clinical practice to utilise routine assessment of ctDNA, there has been significant progress in the development of highly sensitive and reproducible methods (11,12).

Diaz Jr *et al.* found that at baseline, prior to the initiation of panitumumab therapy, 3 of the 4 confirmed KRAS mutant cases had detectable serum levels of mutant KRAS. Where identified, the mutation in serum correlated with

the mutation identified in archival tumour. No serum KRAS mutations were identified in the wild-type cases. Examination of serially acquired serum samples, collected at four-week intervals until progression, revealed nine of the originally wild-type patients developed KRAS mutations detectable in serum; three of whom had multiple mutations. The timing of appearance of these detectable mutations was relatively consistent across the nine patients, averaging 5-6 months. Of the nine patients who had KRAS mutations identified during treatment, three had ctDNA identified before radiographic evidence of disease progression. The lead time between identified ctDNA and radiological confirmation of disease progression averaged 21 weeks (7). This highlights the potential for the early recognition of evolving resistance mechanisms prior to clinically-recognised disease progression and hence opens the possibility of the early incorporation of individualised treatment strategies to further delay or prevent the clinical manifestations of resistance.

To evaluate the tumour evolutionary process and the possibility that KRAS mutations pre-existed in wild-type classified tumours, the authors used mathematical modelling based on the average tumour growth rate derived from their data on ctDNA and known tumour burden combined with theoretical estimates of cell birth and death rates. These results suggested it was highly probable that the KRAS resistant mutations that developed during panitumumab therapy were present in a clonal subpopulation prior to the initiation of therapy. Furthermore, the timing of emergence of resistance reflected the time taken for the resistant subclone to expand, presumably due to a selective growth advantage compared to the sensitive cells (7).

In the same issue of *Nature*, there was a second paper evaluating the role of KRAS mutations in the development of acquired resistance to anti-EGFR therapy, complementing the article by Diaz Jr *et al.* (8). It used an *in vitro* model of acquired resistance, generated by exposing two KRAS wild-type colorectal cell lines to continuous cetuximab treatment. Gene copy number analysis and mutational profiling of the cetuximab-resistant variants revealed both KRAS amplification and mutations. Deep sequencing of the parent cell line revealed that one of the mutations was present in a small proportion of cells. Their findings suggest that resistance observed can be due to selection of KRAS mutant cells as well as the acquisition of KRAS mutations due to the selective pressure exerted by cetuximab treatment. Deep sequencing analysis of tumour tissue biopsied after the development of resistance

to cetuximab or panitumumab therapy and of serial plasma samples during therapy from a small patient set suggested the results identified *in vitro* are clinically relevant mechanisms of acquired resistance.

The implications of the research conducted by Diaz Jr *et al.* is that the development of resistance to anti-EGFR therapy in mCRC is inevitable, due to the presence of pre-existing resistant subclones that expand during targeted therapy (7). It highlights diagnostic and therapeutic issues that are critical to future research evaluating strategies to overcome acquired resistance. Relying on archival tumour tissue from a single time-point to guide genotype-specific therapy may be inadequate due to tumour heterogeneity and the evolution of different tumour cell subclones under selective treatment pressure. The authors presented encouraging results that monitoring ctDNA may be an alternate, minimally invasive way of genotyping tumours at diagnosis and monitoring response to treatment. However, this approach still needs to overcome the technical challenges surrounding the quality and quantity of ctDNA obtained using current methods. There is a need for further research into the relative therapeutic implications of different tumour subclones that may be identified using these novel techniques. The ultimate goal is to enable the rational selection of combinations of targeted treatments or new more effective targeted agents as potential means of delaying or preventing the clinical emergence of resistance.

The potential of using readily accessible plasma samples to personalise and monitor treatment for cancer patients is very exciting. However, we still need to overcome the issues that have long impacted on the use of cytotoxic chemotherapy that in a cubic centimetre of cells there are a billion cancer cells and thus it is highly likely that some are resistant to a particular therapy, molecular targeted or not.

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