

Tumor Evolution as a Therapeutic Target

Nabil Amirouchene-Angelozzi¹, Charles Swanton², and Alberto Bardelli^{1,3}

ABSTRACT

Recent technological advances in the field of molecular diagnostics (including blood-based tumor genotyping) allow the measurement of clonal evolution in patients with cancer, thus adding a new dimension to precision medicine: time. The translation of this new knowledge into clinical benefit implies rethinking therapeutic strategies. In essence, it means considering as a target not only individual oncogenes but also the evolving nature of human tumors. Here, we analyze the limitations of targeted therapies and propose approaches for treatment within an evolutionary framework.

Significance: Precision cancer medicine relies on the possibility to match, in daily medical practice, detailed genomic profiles of a patient's disease with a portfolio of drugs targeted against tumor-specific alterations. Clinical blockade of oncogenes is effective but only transiently; an approach to monitor clonal evolution in patients and develop therapies that also evolve over time may result in improved therapeutic control and survival outcomes. *Cancer Discov*; 7(8); 805-17. ©2017 AACR.

INTRODUCTION

A major issue in the treatment of patients suffering from cancer is the development of resistance to therapies. This ability of cancer to adapt to pharmacologic pressures can be described in terms of tumor evolution, and stems from its intrinsic diversity, or heterogeneity. Tumor heterogeneity refers to the coexistence of cellular populations bearing different genetic or epigenetic alterations within the same lesion, or in different lesions of the same patient. Tumor evolution depicts changes in tumor heterogeneity along the temporal axis and describes the dynamics by which, under environmental pressure, subpopulations of cancer cells bearing selective advantages emerge at the expense of others. This process appears to be particularly marked when cancer undergoes sudden selective pressures imposed by medical treatment.

Recent advances in the longitudinal detection and quantification of tumor-specific mutations in blood, through liquid biopsy, have allowed the definition of patterns of clonal evolution as a measurable characteristic of a patient's cancer. Therapy approaches have so far been based on the characterization of tumors in a two-dimensional way, that is, by means of *in situ* tissue analyses (depicting disease as the sum of

molecular alterations of a particular lesion at a definite time point); now, longitudinal tracking of cancer mutations offers unprecedented opportunities to attempt to modulate tumor evolution for therapeutic purposes.

This review will discuss the relevance of measuring tumor evolution as a readout for response to therapy and the possibility to exploit evolution itself to harness cancer. We will first present the technical approaches that support the measurement of tumor evolution; we will then discuss the role of tumor evolution in the development of resistance to therapy and propose different strategies to exploit the evolving nature of tumors for the benefit of patients with cancer.

TECHNIQUES FOR MONITORING TUMOR EVOLUTION

Multiregion Sequencing

The comparison of synchronous samples derived, in a single patient, from multiple regions of one or more neoplastic lesions led to the notion that solid tumors are genetically heterogeneous, which has been demonstrated across different cancer types through sequencing of spatially separated samples (1-5).

Translating Darwin's evolutionary principles to cancer pathogenesis, tumor heterogeneity has been interpreted as a result of both the acquisition of (epi)genetic variability, fostered by genetic instability, and the selection of distinct subpopulations driven by external pressures, microenvironmental conditions, as well as "mere" geographical factors (neutral evolution; ref. 6). Consequently, information issued from multiregion biopsies could effectively be used to reconstruct the evolutionary dynamics (or "history") of a tumor, graphically rendered as tumor phylogenetic trees, where trunk or clonal alterations, which are present in all

¹Candiolo Cancer Institute, Fondazione del Piemonte per l'Oncologia (FPO), IRCCS, Candiolo, Torino, Italy. ²University College London Cancer Institute and The Francis Crick Institute, London, United Kingdom. ³Department of Oncology, University of Torino, Torino, Italy.

Corresponding Author: Alberto Bardelli, Candiolo Cancer Institute-FPO, IRCCS & Department of Oncology, University of Torino, SP 142 Km 3,95, 10060 Candiolo (TO), Italy. Phone: 39-011-9933235; Fax: 39-011-9933225; E-mail: alberto.bardelli@unito.it

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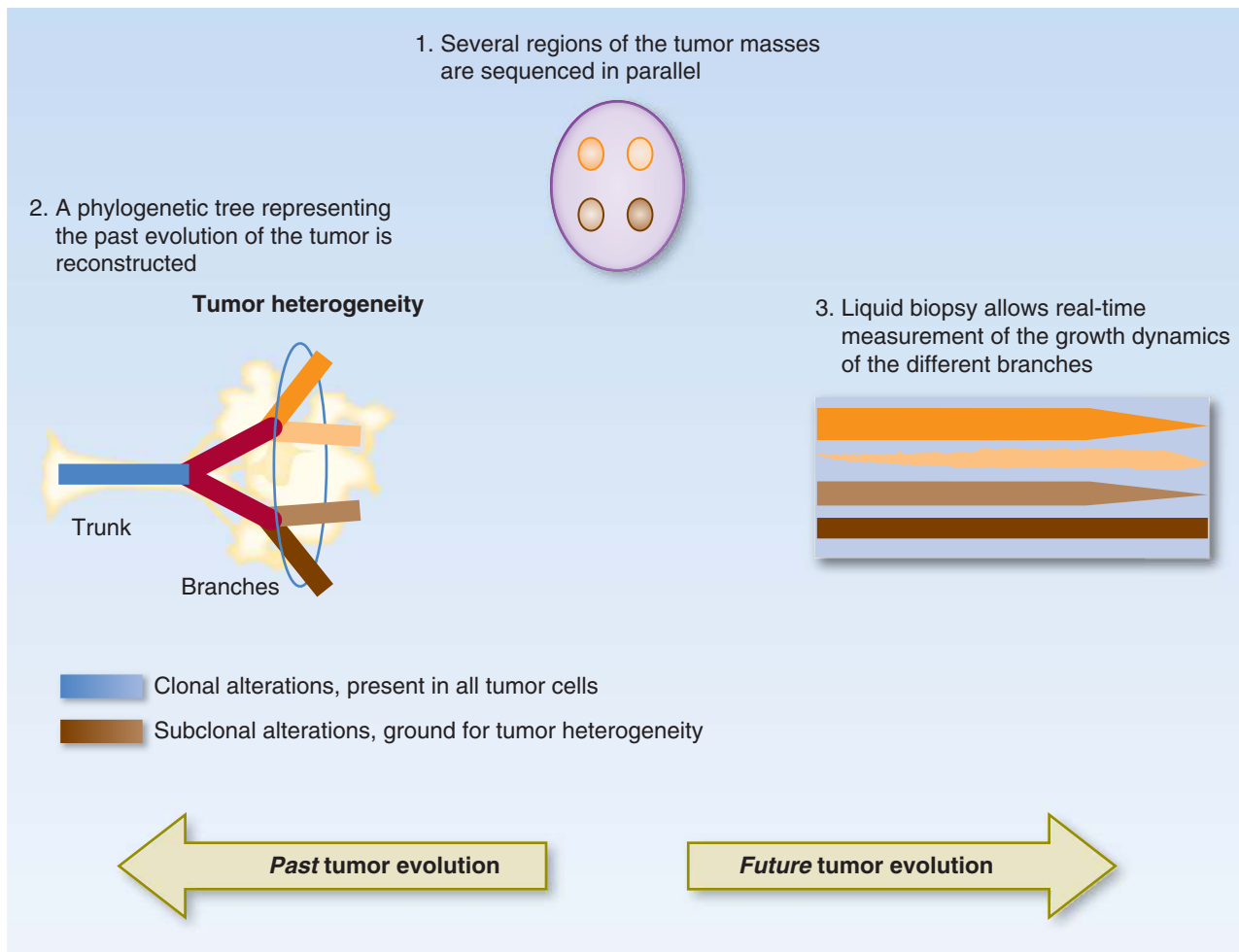


Figure 1. Tracking cancer evolution in space and time. Multiregion biopsy consists of parallel analysis of tissue derived from different regions of a single neoplastic mass, and from distinct metastatic lesions from the same patient (step 1). By assessing their pattern of occurrence in the different samples, clonality of individual alterations is extrapolated. Clonal alterations, present in all samples analyzed (blue) likely represent “ancestral” events, occurred early in tumorigenesis, and are therefore represented as the phylogenetic “trunk” of the tumor (step 2), whereas heterogeneous (subclonal) events (shades of brown) have likely occurred later and therefore represent the “branches” of the phylogenetic tree. Subclonal alterations are the ground for tumor heterogeneity, adaptability to therapy, and cancer evolution. Liquid biopsy allows longitudinal assessment of the growth dynamics of different subclones by cross-comparison of the relative frequencies of mutated subclonal alleles and normalization on (putative) trunk alterations (step 3).

tumor cells, represent ancestral events, whereas heterogeneous genetic alterations constitute the branches (Fig. 1; ref. 7). However, static punctual assessment of multiple but limited tissue samples is not sufficient to fully describe spatial tumor heterogeneity, nor to appropriately describe the profound dynamics of nonhomeostatic biological systems such as tumors.

Liquid Biopsies

As clonal evolution is defined by changes of tumor heterogeneity over both space and time (temporal heterogeneity), its analysis requires the ability to track tumor-specific genetic alterations in real time. Multiregion sequencing proves that single biopsies from geographically localized tumor areas cannot recapitulate the complexity of spatial heterogeneity (1–5). Moreover, morbidity related to the surgical procedure strongly constrains repeated longitudinal sampling, espe-

cially in patients with metastatic disease undergoing therapy, thus limiting reliance on tissue biopsies for the measurement of clonal evolution.

Recently, the increase in sensitivity of DNA-sequencing techniques has allowed genetic characterization of tumors from the analysis of circulating tumor DNA (ctDNA) isolated from plasma and other biological fluids (liquid biopsy; ref. 8). Analysis of ctDNA is based on the identification of tumor-specific alterations, which accounts for its high specificity and sensitivity (9–11) and detection rates comparable with those of tissue biopsies (12–14). Moreover, the half-life of cell-free DNA (cfDNA) being about 2 hours (8), changes in the allelic frequencies of genetic alterations can be monitored in real time. Both clonal and subclonal alterations can be detected by liquid biopsy: “phylogenetic ctDNA tracking” was effectively performed in patients with early-stage non-small cell lung cancer (NSCLC) who underwent multiregion

biopsy sampling and had a selected panel of single-nucleotide variants representative of trunk and branch mutations longitudinally monitored through liquid biopsy (15–17). This shows that information obtained at the time of surgery from multiregion biopsy analysis (on spatial heterogeneity) can be, to a certain extent, translated to ctDNA analysis.

Liquid biopsy allows the tracking of the evolution of different cell subclones (see also Fig. 2), and this was proven to be particularly effective in the follow-up of patients treated with targeted therapy in the metastatic setting. In this setting, the increase in the relative frequency (allelic fraction) of alterations mediating resistance to specific targeted agents has been used to measure the rise (evolution) of refractory tumor subpopulations (branches). In cetuximab- and panitumumab-treated colorectal adenocarcinomas (CRC), for example, blood-based detection of increasing levels of *KRAS*-mutated variants in plasma allowed the identification of resistant subclones before relapse was evident by imaging diagnostics (12, 13); in the same setting, multiple *KRAS* alterations and concomitant *KRAS* and *NRAS* mutations (polyclonal drug resistance) were identified in several studies (9, 18–20). Similar observations have been reported for patients with both breast (21) and lung cancers (22). Exome sequencing can be applied to ctDNA to systematically dissect clonal evolution, as suggested by the study by Murtaza and colleagues, who investigated genetic markers of resistance in a long-term follow-up of patients with breast, ovarian, and lung cancers undergoing different lines of treatment (23). In addition, whole-genome sequencing analysis of ctDNA from the blood of patients with colorectal or breast cancer allows detection of chromosome copy number and structure alterations (24). For example, Shoda and colleagues monitored in plasma the dynamics of *HER2* amplification in a patient with gastric cancer treated with trastuzumab (25), and Liang and colleagues detected, through ctDNA analysis, the coexistence of *EGFR* mutation and *EML4-ALK* gene translocation in a patient with metastatic NSCLC who had relapsed following first- and second-line EGFR inhibition, supporting the effective treatment with two lines of ALK inhibitors (26).

Differences in the amount of ctDNA are related to tumor histologic type, location, and stage and, in addition, in primary NSCLC to necrosis and metabolic activity. Most patients with advanced-stage ovarian and liver cancers as well as metastatic cancers of the pancreas, bladder, colon, lung, stomach, and breast present with measurable ctDNA compared with a minority of patients with medulloblastomas, gliomas, and metastatic cancers of the kidney, prostate, or thyroid (9). The proportion of patients with detectable levels of ctDNA is high in advanced disease but is significantly reduced in early stages (9). However, minimal residual disease monitoring through ctDNA in patients with colon (27, 28), breast (29), and lung cancers (17) has been reported. Diffusion of free tumor DNA is also limited by human anatomy, and transit through the blood–brain barrier is limited. A recent study emphasized that in primary tumors of the brain and brain metastases, the cerebrospinal fluid is a more informative source of ctDNA than plasma (30). Similarly, cfDNA collected from thoracic effusions and malignant ascites might be highly informative: Krimmel and colleagues successfully identified *TP53* mutations in cfDNA from peritoneal fluid from patients with high-grade serous ovarian tumors (31), and bronchoalveolar lavage and

pleural fluids were successfully used to detect *EGFR* mutations in advanced NSCLC (32). ctDNA has also been isolated from saliva and urine (33, 34). Therefore, analysis from multiple sources could be useful in dissecting interlesion heterogeneity. However, although multiregion tissue sampling allows the dissection of spatial heterogeneity, this is impossible with liquid biopsy alone. For example, ctDNA-releasing mutant cells could either be dominant in one or few lesions or consist of smaller populations intermixed within all metastatic sites; in both cases, the resulting circulating mutant allelic fractions could be similar. In addition, factors including size of the lesions, necrosis, and vascularization may also play a role in the relative amount of mutant DNA released in the circulation.

Effective monitoring of tumor evolution would thus require the clinician to carefully select tissue samples to be biopsied (with the specific aim to depict tumor heterogeneity), choose time points for circulating DNA detection, and integrate the molecular scenario with information derived from “standard” techniques, such as imaging diagnostics and protein markers. Comprehensive studies encompassing multiple diagnostic techniques to monitor tumor evolution during treatment, such as TRACERx, recently provided evidence of the feasibility of this approach (16, 17).

TUMOR EVOLUTION IN RESPONSE TO THERAPY

Analysis of posttreatment samples sheds light on how, despite undeniable proof of clinical efficacy of targeted therapies (35–37), with few exceptions (35, 38) the emergence of acquired drug resistance inevitably limits the gains achieved in overall survival with such treatments (39–45). Similarly, markers of increased tumor heterogeneity (the substrate for evolution) have been associated with worse outcome beyond targeted therapy, for example, in head and neck cancers (46), NSCLC (4), ovarian cancer (47), and chronic lymphocytic leukemia (48). Evidence of widespread primary and emerging acquired resistance to immunotherapy (49–52) suggests that at least some tumors are capable of adapting to a therapeutically unleashed immune response. Thus, evolutionary adaptation to therapy appears as a hallmark of cancer, and the possibility to understand and quantify this hallmark highlights the need (and opportunities) for devising cancer therapies aimed at overcoming disease recurrence.

Preexisting Secondary Resistance to Therapy: The Paradigm of Kinase Inhibitors

The possibility of systematically identifying, with high-resolution sequencing techniques, genetic markers (alterations) of targeted drug resistance in relapsed tumors and pretreatment samples has revealed that small populations of genetically resistant cell subclones often already preexist treatment, supporting the idea that clonal selection of preexistent populations is the main mechanism for acquired resistance to targeted therapy (53, 54).

For example, in lung adenocarcinoma bearing activating exon 19 deletions or L858R mutations, the emergence of the *EGFR* mutation T790M is the most common mechanism of resistance to the *EGFR* inhibitors erlotinib and gefitinib (55), and the identification of the T790M allele in pretreatment

samples has been associated with shorter progression-free survival (56). Similarly, amplification of the *MET* oncogene is also detected in 22% of lung specimens developing resistance to EGFR kinase inhibitors (57) and was found in patients and cell lines prior to drug exposure (58). The coexistence in these tumors of different phylogenetic branches characterized by diverse genetic profiles explains how the dynamic balance between different subclones allows tumors to escape even from administration of next-generation inhibitors designed to specifically target resistant cells. Indeed, in tumors treated with osimertinib, one of the third-generation EGFR inhibitors capable of overcoming T790M-mediated resistance (59, 60), not only does the EGFR C797S resistance mutation emerge among T790M-positive clones, but also an increase in the tumor fraction positive for EGFR-activating alterations but lacking T790M mutation has been witnessed (61). Similarly, resistance to the third-generation inhibitor rociletinib may not only be mediated by EGFR (L798I, C797S) mutations, but also by alterations of *MET*, *PIK3CA*, *ERRB2*, and *KRAS* (22), and by the negative selection of T790M-mutant subclones (62).

Analogous observations were made in CRC treated with the anti-EGFR antibodies cetuximab and panitumumab. In this setting, RAS pathway mutations and mutations in the extracellular domain (ECD) of EGFR are predominant resistance mechanisms (12, 13, 19, 20, 63). These mutations often coexist in the same tumor (12, 18, 19), where different cell clones can harbor distinct *KRAS*, *NRAS*, and *BRAF* alterations (13, 18, 19, 64). Moreover, Siravegna and colleagues showed, through liquid biopsy, that upon drug withdrawal, the allelic frequencies of mutated *KRAS* decline in the blood of patients with CRC resistant to anti-EGFR agents (18).

De Novo Acquired Secondary Resistance to Therapy

Mathematical modeling of CRC tumor growth in patients supports the notion that the complex patterns of polyclonal resistance often witnessed in clinical practice are unlikely to originate only, or mainly, *de novo* within the short timeframe of pharmacologic treatment (12). However, although the presence of RAS-mutated clones in CRC resistant to anti-EGFR antibodies is detected prior to treatment in patients and cell lines (13), the same is not observed for EGFR ECD mutations (19, 20, 63), suggesting that these variants might originate primarily upon treatment or be present at such low frequencies prior to treatment as to evade detection by current sequencing technologies. Indeed in patient-derived lung cancer cells treated with gefitinib, Hata and colleagues described both the emergence of early-resistant subclones, derived from preexisting T790M-mutated cells, and the detection of late-emerging resistant populations (65); the latter showed *de novo* appearance of T790M mutation in drug-tolerant, persister cells (66) in which resistance exists at the epigenetic level. Interestingly, these cells appear to be less sensitive to third-generation EGFR inhibitors (65). Moreover, Ramirez and colleagues demonstrated that multiple resistance mechanisms could emerge from a single drug-tolerant clone of PC9 cells sensitive to erlotinib (67), and drug sensitivity of drug-tolerant PC9 cells is restored by IGF1R inhibition (66). *EGFR* ECD mutations were shown by Van Emburgh and colleagues to emerge later in cfDNA when

compared with *RAS* mutations and to be associated with longer progression-free survival in patients with metastatic CRC (68); this observation is consistent with a two-step progression model of *de novo* acquired resistance. Thus, liquid biopsy could possibly be used to identify patients in whom a therapy directed against persister cells might eradicate the reservoirs of drug resistance.

Several reports also highlight that nongenetic mechanisms of resistance are involved in response to targeted therapy and might play an important role in clonal evolution. For example, increased secretion of TGF β and amphiregulin by CRC cells resistant to cetuximab was shown to sustain neighboring sensitive cells (69). A study of 67 secondary resistant melanomas treated with MAPK inhibitors revealed that 39% of cases were not accounted for by any validated mutational mechanism (70), suggesting nongenomic adaptive resistance. In T-cell acute lymphoblastic leukemia (ALL), γ secretase-resistant persister cells were found to be dependent on chromatin regulator BRD4 overexpression, and BRD4 inhibition resensitized cells to therapy (71). Acquired resistance to anti-PD-1 checkpoint inhibitor in NSCLC has been correlated with upregulation of alternative immune checkpoints (50), showing that adaptive epigenetic evolution mediates therapeutic resistance in several settings. Indeed, liquid biopsy might allow effective integration of epigenetic markers by determination of methylation profiles from ctDNA and possibly through the characterization of tumor-derived exosomes (72, 73). Transcriptional analysis of circulating tumor cells is also informative as shown by the identification of noncanonical WNT signaling pathway activation in patients with androgen-resistant prostate cancer (74).

TARGETING CANCER EVOLUTION

If clonal evolution eventually drives resistance to therapy, the possibility to measure it through tissue and liquid biopsy might be pivotal in guiding identification of the most effective additional lines of treatment (Fig. 2). Here, we discuss the rationale, applicability, and possible limits of strategies having as an endpoint the modulation of a tumor's evolution, which are schematically represented in Fig. 3.

Modulating Genomic Instability

Tumor evolution is fueled by (epi)genetic alterations, leading to reduced genomic stability. Examples range from familial and sporadic colorectal cancers with loss of function of mismatch repair proteins, *BRCA1*- and *BRCA2*-deficient breast and ovarian cancers with deficiency in homologous recombination repair, to ultramutated tumors characterized by impaired proofreading activity of polymerase epsilon and delta (75). Recently, activation of APOBEC family proteins has been suggested to increase mutational rate across half of human cancers (76) and to represent a common cause of subclonal diversification in NSCLC (17).

The actionability of molecular alterations in genes controlling genome stability has only partially been tested. A well-known example is the use of a synthetic lethal approach to selectively kill homologous recombination-deficient cells, as demonstrated by the activity of PARP inhibitors in *BRCA*-deficient and *BRCA*-like tumors (77). Interestingly, this

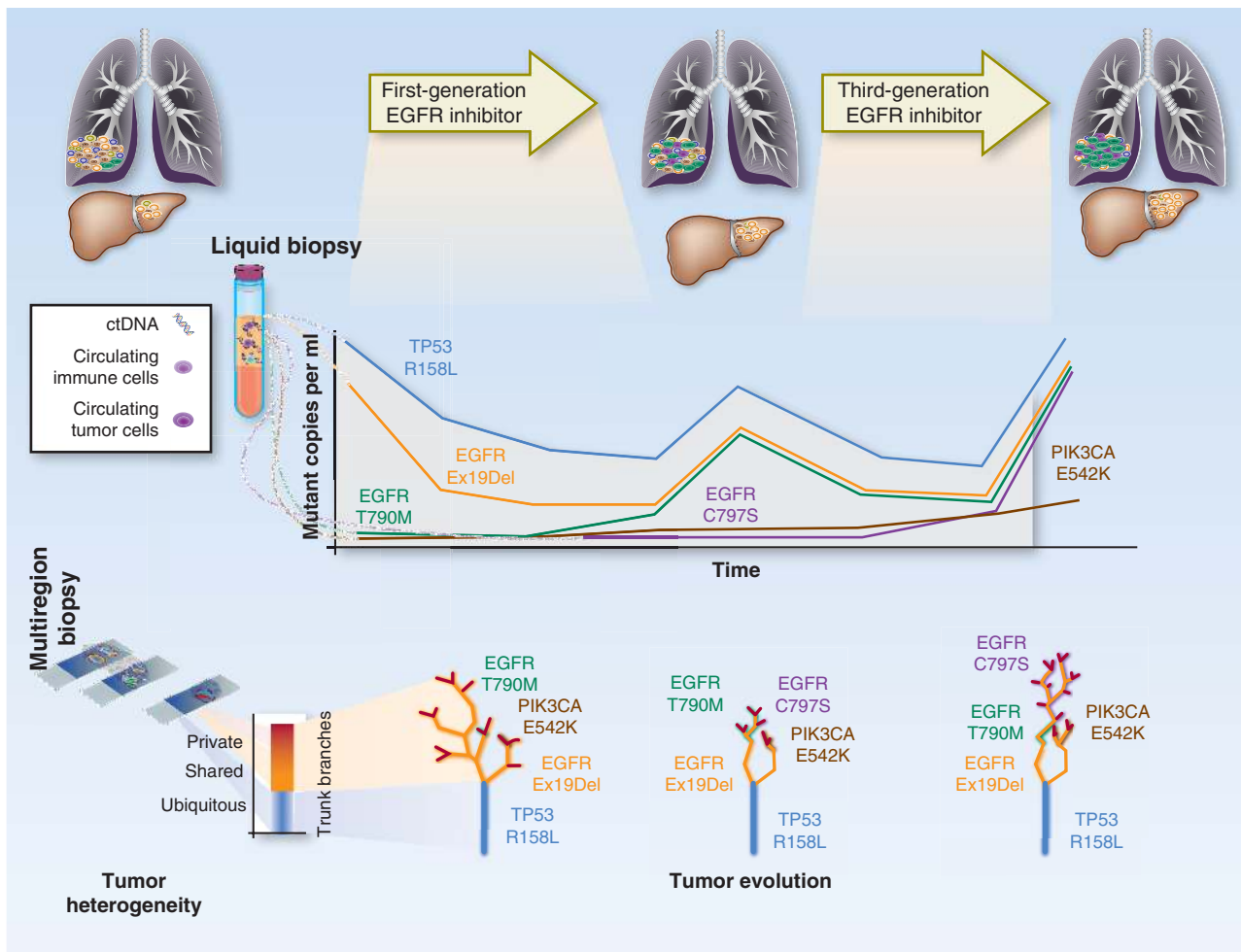


Figure 2. Diagnostic approaches to measure the impact of cancer therapies on clonal evolution. Tumors are molecularly heterogeneous. Multiregion biopsies provide a snapshot of this heterogeneity, allowing the reconstruction of a tumor phylogenetic tree and the identification of ubiquitous, shared, or private alterations. Liquid biopsy allows, through ctDNA analysis, real-time monitoring of changes in tumor heterogeneity under the selective pressure of anticancer treatments. Analysis of the allelic frequencies of subclonal alterations provides a measure of growth dynamics of the different cell populations within a tumor, whereas quantification of trunk alterations allows normalization for tumor burden. Circulating tumor cells could integrate biological information obtained by ctDNA sequencing, and circulating immune cells could help describe the evolution of the tumor-responsive immune microenvironment.

paradigm suggests that increasing genomic instability (i.e., by targeting a complementary pathway of DNA repair) over the threshold of tolerability might lead to a breakdown in genomic integrity, and consequently to cell death.

Moreover, tumors bearing mismatch deficiency show extremely high response rates to immune checkpoint inhibitors and exceptionally long-lasting responses, thus correlating levels of mutational burden with therapeutic efficacy (78). In this regard, increase in the levels of genetic instability could be exploited therapeutically, as suggested by the induction of microsatellite instability (MSI) reported in patients treated with alkylating agents such as temozolomide (79), who might further benefit from immune checkpoint blockade. On the other hand, restraining genomic instability for therapeutic purposes might slow down tumor progression. However, with the exception of p53 loss in preclinical models (80), dependency of tumor cells on specific mutagenic alterations has yet to be proven, and the efficacy of APOBEC

inhibition awaits further validation. It is reasonable to think that such an approach might best affect patients' prognosis in particular in the preventive/adjuvant setting when disease burden and tumor heterogeneity are low.

Targeting Clonal Mutations

As the (epi)genetic heterogeneity of tumor subclones favors evolution under the selective pressure of anticancer drugs, it would be intuitive to think that the administration of drugs targeting truncal alterations present in all cells could better increase the odds of durable control of disease.

In this regard, recent work by Pearson and colleagues has shown that patients with gastric cancer who responded to the FGFR inhibitor AZD4547 harbored tumors with high-level clonal *FGFR* amplifications. In contrast, tumors that did not respond harbored subclonal or low-level amplification (81). In a study of 120 patients with breast cancer undergoing treatment with PI3K/AKT/mTOR inhibitors,

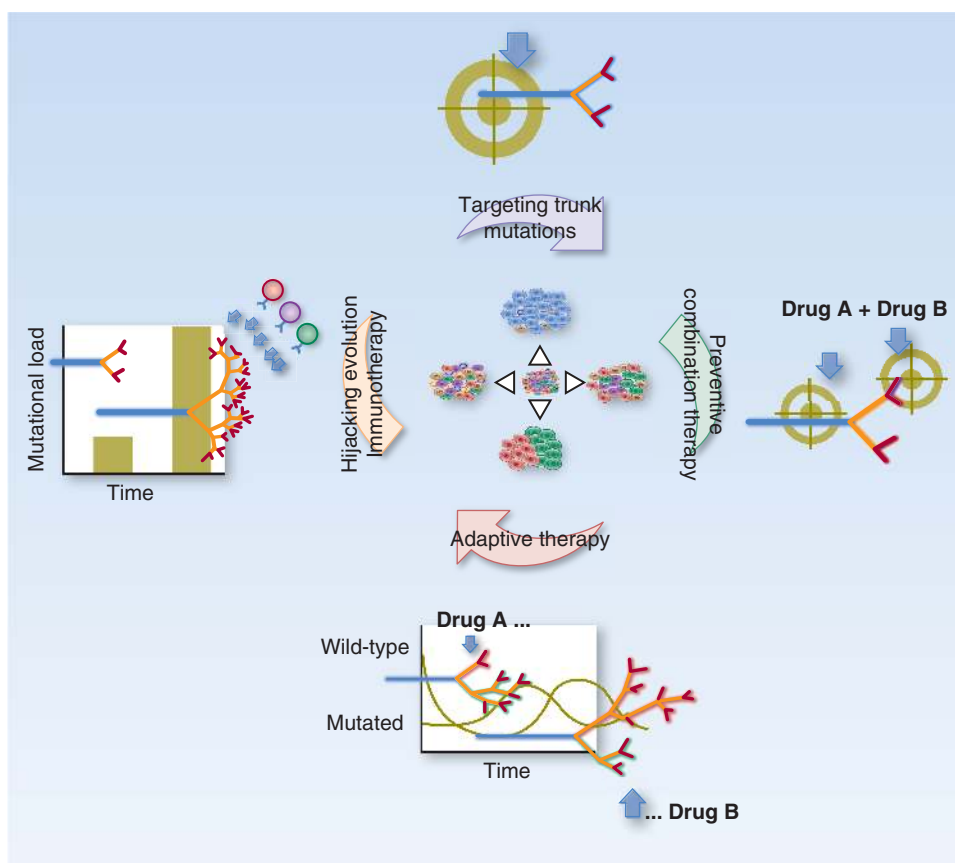


Figure 3. Strategies to target clonal evolution. Measurements of clonal evolution through liquid biopsy (and multiregion biopsy) allow one to select and integrate appropriate strategies to harness tumor evolution. The identification of targetable trunk alterations diminishes the odds of escape of clonal branches lacking the targeted alteration. Preventive combination therapy might allow for extermination of resistant cells before the appearance of further resistance mechanisms. Adaptive strategies like the alternating administration of drug holidays and of treatments targeting different branches of the tumor, coupled with liquid biopsy monitoring of subclonal growth ratios, could foster clonal competition and keep overall tumor growth under control. Immune response could be guided against evolution, either by selection and infusion of tumor lymphocytes specific for trunk alterations, and thus capable to withstand evolution, or by triggering evolution to foster the immune response, by increasing the number of neoantigens.

tumors with clonal *PIK3CA* mutations showed a trend toward better response, which was however not statistically significant (82); indeed, the high frequency of subclonal alterations of PI3K/mTOR across different tumors, as reported in a study based on The Cancer Genome Atlas by McGranahan and colleagues (83), suggests that this could at least partially account for the modest results seen with PI3K inhibitors in patients with solid malignancies (84).

Accordingly, knowledge of the clonal status of actionable drug targets (83) in individual cancers could help the design and implementation of therapies aimed at lowering the odds of acquired resistance. However, direct targeting of clonal alterations is not always feasible: This is the case with loss of function of tumor suppressors such as adenomatous polyposis coli (*APC*). Restoration of *APC* results in induction of apoptosis (85) in colorectal cancer cell lines and tumor regression in preclinical models (86). Unfortunately, pharmacologic restoration of *APC* activity has not yet been achieved. Analogously, restoration of p53, which is significantly enriched in clonal mutations across different tumor types (17, 83), led to tumor regression in autochthonous mouse sarcomas and

lymphomas (80); unfortunately, the actionability of p53 with targeted agents remains challenging (87).

Moreover, aiming at a single truncal oncogenic variant might be insufficient to produce long-term benefit. This has been witnessed in the context of metastatic melanoma, where mutated *BRAF* is a bona fide trunk driver, but therapy with vemurafenib provides only a 2-month increase in overall survival compared with dacarbazine (88). In patients with acquired resistance to *BRAF* inhibition, multiple molecular lesions in MAPK as well as PI3K pathways are commonly detected in the same tumor or among multiple tumors from the same patient (89). Similarly in Ph⁺ ALL, in which *BCR-ABL* translocation is a trunk alteration (90), high rates of relapse to imatinib are observed despite a high initial response rate (91).

Combinatorial Approaches

Empirical associations of multiple effective drugs largely support the effectiveness of chemotherapeutic regimens in both hematologic and in solid malignancies (92, 93). Conceptually, the same paradigm might be applied to targeted

drugs such as inhibitors of oncogenic signaling pathways. Drug association is further sustained by mathematical modeling of acquired resistance; for example, studies on patients with pancreatic cancer, colorectal cancer, or melanoma suggest that, in metastatic cancer, monotherapy with targeted agents cannot eradicate the disease, even in the presence of limited tumor burden (range, 8.5×10^8 – 1.2×10^{11} cells), whereas dual combination therapy offers hope of success only for low tumor burden and in the absence of cross-resistance mutations (94). Therefore, three or higher order combination therapies might be needed to obtain tumor eradication even with agents targeting truncal alterations; analogously, inhibition of distinct pathways would also be required to avoid cross-resistance. Such combinatorial approaches will likely be limited by the number of therapies available targeting multiple distinct clonal alterations and toxicity to normal tissues.

Targeting Trunk Mutations with Immunotherapy

As previously discussed, affecting multiple clonal alterations with targeted agents is limited by druggability and toxicity issues. A strategy to overcome these limitations involves targeting clonal neoantigens, or dominant branched antigens that were selected through evolutionary bottlenecks such as surgery or systemic therapy, through personalized vaccines or adoptive cell therapy (95). The possibility to target multiple neoantigens through these approaches would significantly reduce the odds of resistance. The latter has been shown to be associated with loss of expression of neoantigens (either by genetic or epigenetic mechanisms) in 2 patients with melanoma who underwent T-cell adoptive infusion (96). Moreover, in patients with ALL who responded to CART-19 infusion, mechanisms of resistance implicated acquired mutations but also alternative splicing of immunogenic epitopes (97). In this context, liquid biopsies could be particularly effective in tracking dynamics of the targeted neoantigens, and, coupled with T cell-receptor sequencing from blood, in predicting the odds of relapse (98). However, immune evasion from T cells aimed at clonal neoantigens could, for example, arise through clonal selection of tumor cells bearing mutations or loss of HLA (99, 100); the latter was recently reported in a patient with metastatic CRC who relapsed after adoptive T-cell transfer (101); alterations of IFN γ pathway effectors could also impair a targeted T cell-mediated immune response (51). This suggests that immunotherapy alone might not be sufficient to eradicate a tumor, and integration with other forms of therapy coupled with diagnostic monitoring of tumor evolution might be needed to maximize efficacy.

Preventive Combination Therapy

The observation that resistant cell clones often preexist (although undetectable) at the start of treatment supports the idea that early administration of combinatorial treatments stands a higher chance of eradicating such clones when their number is very low, before acquired resistance is overtly diagnosed. *Ab initio* combination therapies are particularly effective in preventing resistance in other pathologic contexts, such as infectious diseases (102). In this setting, drug combinations have proven effective against fast-evolving pathogenic agents, such as HIV (102). In oncology, however, the narrower therapeutic window between tumor cells and host poses limits to the number of agents that can be simultaneously combined. Liquid biopsy

sequencing could guide evolution-based combination regimens aiming initially at reducing the odds of resistance and further exploiting escape mechanism to maintain tumor growth control when resistance develops. Targeted drug association *ab initio* could aim at simultaneously targeting the bulk tumor (with a drug active on the trunk) and the expected secondary resistance mechanism, thus providing a significant advantage in survival compared with administration at relapse (94).

Acquired resistance mediated by the emergence of secondary mutations of the drug target has often been witnessed with imatinib, dasatinib, and nilotinib in chronic myeloid leukemia and with different generations of EGFR inhibitors in lung cancer and suggests that reactivation of the inhibited pathway is a biologically favored mechanism (103, 104). Similarly, 14 different metastatic lesions from a patient with breast cancer bearing an activating *PIK3CA* mutation who relapsed under therapy with the PI3K α inhibitor BYL719 bore different *PTEN* genetic alterations, resulting in convergent loss of *PTEN* expression, which was reverted, in the corresponding patient-derived xenograft, by simultaneous PI3K p110 β blockade (105). Indeed, the observation that often, upon inhibition of an oncogenic driver, a relevant number of escape mechanisms converge on that same pathway suggests that at least in certain tumors preventive combination therapy providing vertical inhibition of a trunk target and its downstream effectors might reduce the probability of relapse (i.e., delay it). Moreover, synchronous targeting of downstream players of drug resistance would not just represent a preventive action but could also result in increased cytotoxic effects on the bulk of the tumor and thus in deeper reduction in tumor burden. This could also limit reservoirs of *de novo* resistance.

In CRCs, for example, secondary resistance to anti-EGFR antibody therapy, which interferes with signaling through the MAPK cascade, is often mediated by reactivation of the pathway through additional gain-of-function alterations in *RAS*, *MEK*, and *MET* (54, 106). Following these observations, Misale and colleagues demonstrated that combinatorial treatment of EGFR-sensitive colorectal cancer models with vertical inhibition of EGFR and MEK (which is a downstream effector of MAPK pathway) prevents the occurrence of resistance (107), and a clinical trial adopting this approach in EGFR-sensitive CRC is ongoing to test the hypothesis (EudraCT 2014-002460-33). Similarly, combined EGFR/MEK inhibition was reported to prevent emergence of resistance in *EGFR*-mutated lung cancer models (108). Crystal and colleagues described the establishment of a platform of patient-derived models of acquired resistance for the identification of effective targeted drug combinations: Cell lines were derived from patients with lung cancer, made resistant to single-agent inhibition of a primary driver oncogene, and screened for agents capable of overcoming resistance. Selected compounds were tested in mice. Notably, combination treatments *ab initio* showed increased response compared with combinations administered at resistance (109).

A limit to this approach is the variability of resistance mechanisms witnessed in patients: The combination of BRAF and MEK inhibitors results in increased survival in patients with melanoma (ref. 110; whereas sequential therapy showed no such benefit; ref. 111); nevertheless, tumor relapse is observed (112). Indeed, in metastatic melanoma that lost sensitivity to MAPK inhibitors, not only alterations of *BRAF*,

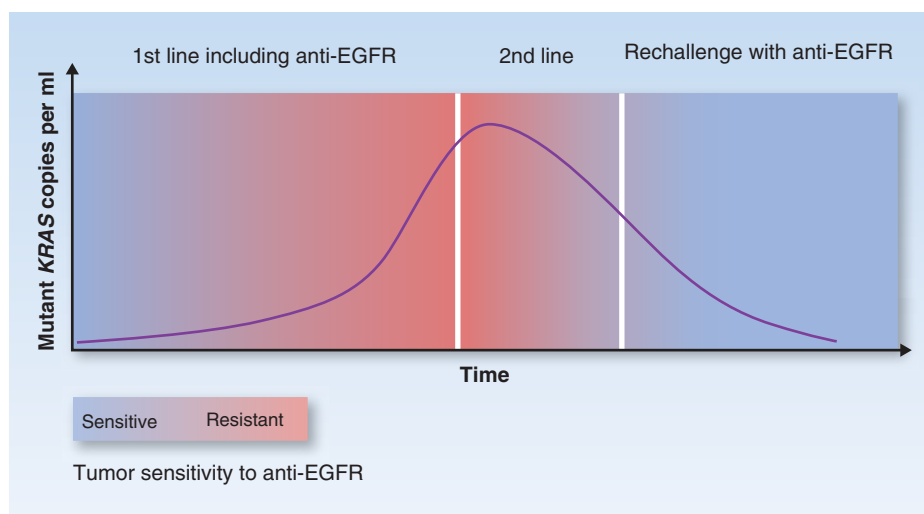


Figure 4. Liquid biopsy as a tool to guide adaptive treatment approaches. In CRCs that respond to treatment with anti-EGFR antibodies, *KRAS* mutations often emerge as a mechanism of acquired resistance. Notably, *KRAS* mutations decline in ctDNA when EGFR blockade is suspended (18). Monitoring the evolution of *KRAS*-mutant alleles in ctDNA can be used to design additional lines of therapy aimed at rechallenging patients who initially respond to and then relapse on EGFR blockade.

NRAS, *KRAS*, *MEK1*, or *MAP2K1* are enriched at relapse but also activation of divergent escape pathways, as suggested by the evidence of gain-of-function events in *PIK3CA*, *AKT1*, and *AKT3* and loss-of-function events in *PIK3R2*, *DUSP4*, *CDKN2A*, *PTEN*, and possibly nongenomic alterations such as *MET* overexpression, and β -catenin and *YAP1* deregulation (70). Recently, combinatorial agents capable of preventing divergent bypass escape mechanisms were tested in the form of antibody mixtures. For example, the EGFR antibody mixture Sym004 was shown to overcome cetuximab resistance mediated by EGFR ECD mutations in CRC (113), and pan-HER, targeting EGFR, HER2, and HER3, was shown to act synergistically on tumor cells, possibly preventing bystander resistance due to compensatory activation of EGFR family receptors and increased production of ligands (114, 115).

Of note in this setting, the levels of heterogeneity might be systematically underestimated in preclinical models, where the reduced number of cells (compared with a patient with metastatic disease) implies a parallel reduction in heterogeneity. Thus, comprehensive integration of data from multi-region and liquid biopsies in large cohorts of patients should guide the definition of effective preventive combinations with two or more drugs, and lead to the identification of more favorable clinical conditions where higher efficacy could be achieved (e.g., after debulking surgery).

Adaptive Therapy

Cell-specific fitness in the presence of therapy could also be exploited to harness tumor evolution. Resistance may come at a fitness cost and subclones showing a growth advantage under targeted therapy could lose their fitness advantage in the absence of the selective pressure. In patients with CRC who became resistant to cetuximab or panitumumab and showed emergence of a *KRAS*-mutated cell population, the interruption of therapy or treatment with a different class of compounds (e.g., a VEGF inhibitor) correlated with a reduction of *KRAS*-

mutant allelic fraction in plasma, as assessed with liquid biopsy (18, 116). In a patient-derived xenograft model of melanoma displaying resistance to BRAF inhibition, intermittent dosing of vemurafenib led to long-term control of tumor growth, unlike continuous treatment (117), in line with the observation of responses upon rechallenge in patients with melanoma with acquired resistance to BRAF inhibitors (118, 119).

Mathematical analysis of tumor evolution further supports these observations. Gatenby and colleagues modeled clonal dynamics in the presence or absence of treatment, showing that although high-dose regimens lead to rapid expansion of resistant populations (competitive release; ref. 120), modulation of therapy (adaptive therapy; ref. 121) allows the control of tumor growth. This is achieved by keeping a balance between drug-sensitive tumor cells, which proliferate better in the absence of drug, and resistant cells, which prove fitter only in the presence of the drug itself, as exemplified in ovarian and breast cancer xenograft models (121–123).

Competition between tumor clones could be therefore exploited for therapeutic purposes. In this setting, measurement of clonal evolution through liquid biopsy could guide precise administration of drug holidays and rechallenge (Fig. 4). However, the preexistence of resistant clones prior to therapy suggests that, albeit at different rates, both drug-resistant and drug-sensitive populations are able to proliferate in a drug-free environment, thus supporting the introduction of sequential treatment strategies at molecular relapse. With this perspective, the arising polyclonal multigenic mechanisms of resistance could be turned by liquid biopsy into a therapeutic opportunity for adaptive therapy, allowing the possibility to fine-tune intratumor clonal competition and enforce tumor growth control by alternating two (“evolutionary double bind”; ref. 124) or more drugs specific for different tumor branches (and resistance mechanisms).

A complementary strategy to high-dose alternating regimens implies the administration of reduced doses of therapy (the amount needed to achieve control of tumor growth

rather than the MTD) on a continuous schedule (metronomic therapy). In chemotherapy-treated breast cancer xenografts, metronomic therapy allowed better control of tumor growth than full-dose therapy (122). In this model, intermittent doses (i.e., drug holidays) failed to control tumor growth (122). Indeed, low-dose maintenance regimens have long been evaluated in clinical practice (125); however, evidence showing the control of tumor evolution through metronomic approaches in patients is lacking. In this setting, measuring tumor evolution could offer additional criteria to assess the efficacy of complex regimens and possibly to model more effective sequences of induction and maintenance therapy for solid tumors. The ethical and clinical challenges of adopting novel clinical trial paradigms implementing evolutionary modeling, superseding current approaches of treatment until progression of disease, should not be underestimated.

LEVERAGING BIOMARKERS AND REAL-TIME ASSESSMENT OF TUMOR EVOLUTION

How then should medical intervention be guided? (Precision) cancer medicine has relied so far on predictive biomarkers to help the choice of the most effective therapeutic regimens. The impact of at least partially forecasting tumor evolution (that is, to be able to predict the tumor's next moves) is suggested by evidence of parallel evolution in cancer (126). The ways tumors evolve could be relatively limited even across (epi)genetically different tumors. As already discussed, a definite selective pressure could result in the deregulation of a common pathway. In this regard, in the same patient, genetically distinct subclones often harbor genetic alterations targeting the same gene or pathway through parallel evolution (3, 127–129).

Exhaustive follow-up studies coupling multiregion biopsies with ctDNA analysis and analysis of tumor heterogeneity through autopsy analysis will define to what extent tumor evolution might be predictable (16, 17), and hopefully these studies will provide a comprehensive understanding of the “evolutionary rulebook” of cancers, by distinguishing driver events that are always clonal from those that are often or rarely clonal, by estimating frequencies and dynamics of driver alterations across molecular subtypes, and possibly by revealing how molecular profiles associate with evolution patterns under the pressure of anticancer therapy.

Meanwhile, clinical and preclinical studies testing the efficacy of drug combinations targeting simultaneously the bulk tumor (therefore having a bona fide ubiquitous target) and resistance mechanisms will deliver valuable information to identify associations to administer *ab initio*. For example, in a patient with cetuximab-resistant CRC described by Russo and colleagues, the combination of anti-EGFR antibody panitumumab and the MEK inhibitor trametinib was effective on an MEK-mutated metastatic lesion (but not on a KRAS-mutated clone; ref. 64). Another study coupling functional analysis and tissue genotyping revealed that MET amplification conferred resistance to the combination of panitumumab and vemurafenib in a BRAF-mutated CRC. In the same patient, a combination of vemurafenib with the dual ALK/MET inhibitor crizotinib was capable, even if temporarily, of overcoming resistance (130); interestingly, the choice was supported by pharmacologic analysis on a BRAF-mutant cell line made

resistant to BRAF inhibition and showing emerging MET amplification. Similarly, in the study described by Crystal and colleagues, sequencing of patient samples alone was not sufficient for the prediction of effective combinations, suggesting that patient-derived avatars could also be exploited to functionally define patient-specific drug associations (109).

Further preclinical insight into specific patterns of evolution could point out ways to steer tumor evolution toward more favorable or more targetable molecular backgrounds, for example T790M mutations in lung cancer, which renders resistant clones sensitive to third-generation inhibitors, or EGFR ECD mutations in CRC, which are effectively targetable with the oligoclonal antibody MM-151 (20, 113). Steering tumor evolution could be possibly achieved by targeting specific phenotypes correlated with the “unwanted” genotypes. As an example, the recently reported increased dependency of KRAS-mutated clones on mitochondrial metabolism (131) and on increased uptake of dehydroascorbate (132) could be exploited to decrease the odds of emergence of KRAS-mediated resistance. Longitudinal analysis with liquid biopsy could then enable in patients real-time monitoring for the emergence of the desired phenotype.

It is important to underscore that several technical issues, such as the ability to query spatial and temporal heterogeneity, presently limit our capability to foresee tumor evolution. Moreover, every tumor is unique, even when only genetic alterations are considered (133), and stochastic events might constitute an intrinsic barrier to the prediction of specific mechanisms of drug resistance (134). The clinical implementation of liquid biopsies could provide real-time assessment of tumor evolution, thus allowing a physician to undertake appropriate therapeutic measures and choose the best strategy to harness the evolution of a patient's tumor. Multiple parameters, such as specific markers of susceptibility/resistance to targeted therapy, as well as proxies of response to immunotherapy, could simultaneously be evaluated and possibly be held as endpoints for therapeutic success alongside standard imaging-based parameters.

FUTURE DIRECTIONS

The study of tumor evolution through multiregion sequencing and liquid biopsy has shed new light on our understanding of the neoplastic process and of the mechanisms by which tumors escape to therapy. Progress in these areas will likely be fostered by technological advances and decrease in the costs of sequencing. Clinical application of multiregion biopsy can be supported by single-cell analysis (135), which could provide high-resolution readout of tumor heterogeneity even with limited sampled material. Reduced sequencing costs and increased accessibility to standardized platforms will further foster implementation of liquid biopsies in clinical practice. Moreover, the evidence of epigenetic drivers of targeted therapy resistance (70) as well as the need for the evaluation of the tumor (micro)environment for the follow-up of response to other classes of therapeutics (e.g., immunotherapy) calls for the development of new ways to exploit ctDNA. Transcriptional analysis of tumor RNA retrieved from exosomes, or from circulating tumor cells, could widen our ability to identify and target nongenetic drivers of tumor evolution, and further studies are needed to assess the value of such approaches. Genetic analysis of T-cell receptors is being exploited in an attempt to

trace the “evolution” of T lymphocytes in response to tumor evolution itself (136, 98, 137). Thus, recognition of patterns of tumor progression through multiregion biopsy and liquid biopsies might provide new therapeutic strategies tailored to cancer evolution and tumor–microenvironmental background in individual patients. The design of clinical trials comparing liquid biopsy–driven therapeutic decisions to standard algorithms will be pivotal to promote progress in this area.

Disclosure of Potential Conflicts of Interest

C. Swanton has ownership interest (including patents) in Achilles Therapeutics, APOGEN Biotech, Epic Biosciences, and Grail; has pending patents on Method of detecting tumour recurrence (1618485.5), Method for treating cancer (PCT/EP2016/059401), and Immune checkpoint intervention in cancer (PCT/EP2016/071471); and is a consultant for/received speaker fees from Novartis, Eli Lilly, Roche, Pfizer, Celgene, Boehringer Ingelheim, and Servier. A. Bardelli is a consultant/advisory board member for Horizon Discovery and Biocartis, and is a consultant for/received speaker fees from Novartis, Roche, and Illumina. No potential conflicts of interest were disclosed by the other author.

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