



Future perspectives from lung cancer pre-clinical models: new treatments are coming?

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Abstract: Lung cancer currently stands out as both the most common and the most lethal type of cancer, the latter feature being partly explained by the fact that the majority of lung cancer patients already display advanced disease at the time of diagnosis. In recent years, the development of specific tyrosine kinase inhibitors (TKI) for the therapeutic benefit of patients harboring certain molecular aberrations and the introduction of prospective molecular profiling in the clinical practice have revolutionized the treatment of advanced non-small cell lung cancer (NSCLC). However, the identification of the best strategies to enhance treatment effectiveness and to avoid the critical phenomenon of drug tolerance and acquired resistance in patients with lung cancer still remains an unmet medical need. Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) are two complementary approaches to define tumor heterogeneity and clonal evolution in a non-invasive manner and to perform functional studies on metastatic cells. Finally, the recent discovery that the tumor microenvironment architecture can be faithfully recapitulated *in vitro* represents a novel pre-clinical frontier with the potential to optimize more effective immunology-based precision therapies that could rapidly move forward to the clinic.

Keywords: Circulating tumor cells (CTCs); circulating tumor DNA (ctDNA); tumor organoids; EGFR-mutant NSCLC; KRAS^{G12C} inhibitors; drug tolerance

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Towards the sunset of the traditional biopsy era: the future is liquid

The development of personalized medicine for cancer patients relies on the unambiguous identification of the molecular drivers of their disease (1,2). Currently, tumor biopsy samples are the major source of material for biomarker measurement in order to predict response to therapy (3). This approach is biased by at least three

different aspects. First, since tumor tissue is unavailable or insufficient for genetic analysis at the time of progression in a significant percentage of advanced NSCLC patients (4), treatment decisions, even in the metastatic setting, are often based on primary tumor biopsies diagnosed years before, frequently leading to treatment failure. Second, serial invasive biopsies to follow tumor progression in space and time are often impractical, uncomfortable and,

occasionally, anatomically challenging and associated with some risk, especially in lung cancer patients (5). Third, it is now clear that, given a profound intratumor heterogeneity, a single-site biopsy is very unlikely to capture the complexity of the entire genomic landscape of a tumor (6-10). Next generation sequencing (NGS) has provided robust evidence that both primary tumors and metastases undergo a continuous process of clonal evolution under the selective pressure of anti-cancer drugs (11). Late recurrences occur upon tumor dormancy, whereby tumor cells previously disseminated to distant organs remain in a dormant state while accumulating enough epigenetic and genetic modifications until eventually giving rise to overt metastases (12). Within this framework, one of the main reasons for systemic cancer treatment failure is considered to be our inability to accurately determine spatial and temporal heterogeneity along tumor evolution. It is therefore questionable whether a diagnostic biopsy sample truly represents the disease, particularly when considering options for second line therapies and beyond. Although new classes of anti-cancer drugs, such as TKIs against *EGFR*, *ALK*, *ROS-1*, *RET* and *BRAF* mutations, and checkpoint inhibitors have led to a significant improvement of the prognosis for a selected group of NSCLC patients, resistance towards targeted therapies normally emerges within one to two years, regardless of the line of therapy (13,14). This frequent yet unfavorable outcome highlights the pressing need for novel biomarkers than can trace and predict the genotypic and phenotypic evolution of cancer cells underlying resistance mechanisms to an expanding repertoire of targeted drugs. Therefore, monitoring the disease via minimally invasive techniques in order to adjust treatment at the earliest moment has recently become the main goal for the upcoming standard of care. There is hope that repeated liquid biopsies performed at different time-points can complement traditional biopsy sampling and might be a way to better address the above challenges. This possibility is supported by recent technological advances in the expanding fields of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) which we are going to discuss hereinafter.

ctDNA and CTCs: two complementary strategies to decipher the complexity of advanced cancer

Increasing interest in the exploitation of ctDNA as a tool for early cancer detection and improvement of therapeutic outcomes derives from the recent advances in DNA

sequencing technologies and from a better understanding of tumor molecular biology. ctDNA also shows promising applications in adjuvant therapy by precocious recognition of patients at high risk of recurrence based on the detection of post-surgical minimal residual disease (MRD), thus optimizing risk stratification (15). Cancer patients with an array of different solid malignancies display higher levels of ctDNA compared to healthy individuals, whereby fragmented DNA (typically around 160–180 bp) enters the circulation upon tumor cell apoptosis and/or necrosis (16,17). Mutations can be identified in a wider proportion and with higher sensitivity in ctDNA rather than CTCs (18,19). Even in the absence of detectable CTCs, it is possible to find ctDNA at levels that tend to increase along with disease stage (20,21). Notably, detection of tumor-specific mutations in plasma, including those targeted by TKIs, can avoid invasive and less representative tumor biopsies. Thus, the field of ctDNA as a potential biomarker is being actively explored. Expected implications span from cancer diagnosis to prognosis, treatment selection and monitoring of disease burden (22). Several tumor-specific aberrations can be followed in ctDNA, encompassing somatic single nucleotide variants (SNV), chromosomal rearrangements and epigenetic alterations, and may be even complemented by more comprehensive whole-exome NGS, providing unparalleled sensitivity and specificity as a tumor biomarker (23). To date, ctDNA has resulted as a promising biomarker for the early detection and localization of cancer (24,25) and for the assessment of post-surgical MRD (26-28). Furthermore, plasma samples collected right before drug administration may reflect overall tumor burden, whereas an increase in ctDNA levels immediately after treatment may work as an early indicator of tumor cell eradication and response to therapy. Besides its potential as a biomarker for treatment response, quantitative determination of ctDNA could also be considered a good prognostic indicator (23). Notably, given the limited fraction of tumor-specific DNA in the background of normal circulating free DNA (cfDNA), ctDNA analysis is challenging and requires highly sensitive techniques. Artefactual errors accumulate during library preparation and sequencing when applying NGS technologies to reveal low-frequency mutations, determining a number of false positive calls and sometimes masking the true biological variants (29-31). For this reason, the background error rate should be taken into account by mutation-calling algorithms, which should be as rigorous as possibly achievable to guarantee specificity, sometimes even at the expense of sensitivity for low-frequency

mutations. Another important parameter is the physical limit of detection of a ctDNA analysis platform, which will depend on the frequency at which a rare DNA molecule present in plasma is captured in a blood draw, incorporated into the deriving library and finally sequenced from it (15). Further drawbacks come also from the biological challenges associated with ctDNA analysis. Indeed, not all the true mutations detected in plasma are necessarily specific of cancer cells. Actually, the vast majority of cfDNA is released by hematopoietic cells and progenitors (32,33), which accumulate mutations with ageing without leading to any neoplastic transformation (34,35). Therefore, deep sequencing of both cfDNA and white blood cell DNA might be required in parallel to uncover and filter out all non-cancer related mutations and reduce false positive ctDNA detection. Given the rapid evolution of this research field, ctDNA might be even employed, in the future, as a biomarker for cancer screening programs, as long as the available analytical techniques will reach the highest levels of sensitivity, specificity and reproducibility to achieve this goal.

Complementary to ctDNA, another option for liquid biopsy and pre-clinical evaluation is the analysis of CTCs in the blood, shed from either the original tumor or its metastases. CTCs can be captured from a minimally invasive blood test, are readily amenable to serial sampling and may provide essential insights into intratumor heterogeneity and tumor evolution (36). In patients with advanced cancer, the definition of 'CTC traffic' refers to the process of cell migration in between the primary tumor, bone marrow and metastases (37,38). CTCs originating from distinct sites better represent tumor heterogeneity than any single tumor biopsy. Also, due to the tumorigenic potential of at least a subset of CTCs, these cells are definitely the most critical to characterize, target and ablate. CTCs have been demonstrated to be a robust prognostic marker in multiple cancer types, including lung cancer, and their presence correlates to the metastatic tumor burden and is associated with overall survival (OS) (39-43). Beside their prognostic value, CTC eradication might be considered as an early signal of drug activity and/or could accelerate drug development and guide therapeutic choices. Beyond enumeration, there is growing interest in the genotypic and phenotypic characterization of CTCs, which give the unprecedented opportunity to explore tumor-initiating capacity and metastasis biology *ex vivo*. Early molecular profiling efforts on CTCs focused on technical feasibility as well as comparison of mutational

patterns of CTCs versus primary tumors. Specifically, gene-expression studies turned out key to link phenotypic differences with genetic and epigenetic variations. However, RNA preservation is technically more challenging than DNA, raising initial concerns about the true impact of sample processing on CTC expression profiles and data reliability in terms of biological relevance and influence of background noise (44). Upon the most recent technical advances in single-cell sequencing (45,46), DNA and RNA profiles of CTCs can now be analyzed with a high confidence rate to evaluate the degree of heterogeneity between individual cells and to interrogate potentially actionable targets and emerging resistant subclones, thus supporting patient stratification before treatment. To date, multiple investigators have demonstrated the feasibility of mutational analysis (47), RNA-sequencing (48,49), whole exome-sequencing and somatic SNV analysis (50,51) on CTCs. Although not yet ready for routine clinical applications, the deriving 'signatures' provide useful insights into the molecular characteristics of various tumor types, pointing to the selection of drugs that specifically target the vulnerabilities emerging from these studies. Another unique possibility offered by CTCs in terms of pre-clinical evaluation is the chance to characterize their phenotype and even culture them and verify their drug susceptibility *ex vivo* (52). Stable CTC lines were established from estrogen receptor-positive breast cancer, which were subsequently genotyped and screened for sensitivity to a panel of single drug and drug combinations, including standard clinical regimens and experimental agents targeting specific mutations. The results of the combination of genotyping and functional testing for drug susceptibility turned out essential to define therapeutically relevant driver mutations in view of improving treatment efficacy (52). Furthermore, CTCs allow investigators to explore the biological processes underlying the invasion and metastasis processes, such as epithelial-mesenchymal transition (EMT) (53) and circulating tumor microemboli (or clusters) (54) in a clinically relevant model. Interestingly, some of the first studies to address the EMT phenotype of CTCs were conducted in patients with small cell lung cancer (SCLC) and NSCLC, displaying heterogeneous expression of EMT markers, suggestive of an intermediate rather than a full-scale phenomenon (55,56). An example of a pre-clinical approach with potential consequences on therapeutic decisions comes from the thorough characterization effort conducted on CTC clusters derived from both patients and mouse models in breast cancer. Consecutive studies

revealed (I) the nature of cell-cell interactions within CTC clusters and their contribution to the metastatic spread of cancer (54), (II) the occurrence of specific changes in DNA methylation that promote stemness and metastasis and point to cluster-targeting compounds to suppress cancer dissemination (57), and (III) the association of neutrophils with CTC clusters, which promotes cell cycle progression, increases the metastatic potential of CTCs and suggests the introduction of drugs targeting this interaction for the clinical benefit of patients (58). The main technical challenge when working on CTCs, common to each of the applications discussed so far, is the low abundance of tumor cells in the bloodstream, with estimates of just one CTC per $\sim 10^6$ – 10^7 white blood cells/ml of blood. Multiple technologies for CTC isolation have recently emerged, combining specific methods of enrichment (mostly based on physical properties of the cells or biological features, such as capture through tumor cell surface markers), and detection by immunostaining and microscopy or by PCR-based methods (59,60). Notably, if enrichment is based on preselected markers, CTC heterogeneity might not be fully appreciated. Nevertheless, isolating enough cells is necessary to perform functional tests despite the obvious limitation in the blood volume that can be drawn and analyzed. Over 1,500 reports on CTCs published in the last decade cover the aspects discussed so far, ranging from technology development, to prognostic and pharmacodynamic biomarker applications, as well as identification of predictive biomarkers for treatment selection (36). It is even possible that in the future CTCs might be evaluated as a tissue source in preventive molecular screening programs.

Insights into the potential of ctDNA in lung cancer: from pre-clinical evaluation to clinical practice

The concentration of ctDNA in plasma has been shown to correlate with tumor size and stage, supporting an association between ctDNA levels and prognosis in several malignancies (22). Recent studies have demonstrated that ctDNA analysis allows the non-invasive assessment of emerging mutations associated with treatment resistance directly from plasma DNA and, in parallel, whole exome sequencing can provide a comprehensive assessment of genomic changes including chromosomal aberrations, focal amplifications and gene rearrangements at baseline and upon acquisition of treatment resistance (22,61). In advanced stage cancer, this approach could be beneficial to study

resistance mechanisms *in vivo* and to investigate strategies to overcome or circumvent resistance with specific drug combinations. As previously mentioned, ctDNA may also be used as a biomarker after treatment to identify patients at risk of relapse, for example by monitoring individuals following surgical resection for the early assessment of residual disease (62) or to detect disease recurrence (63,64), which could allow the immediate introduction of second line treatment strategies when the disease burden is still limited or the optimal selection of adjuvant therapy. Patient-derived ctDNA allows investigators to rapidly switch from pre-clinical evaluations to actual therapeutic decisions, crossing the border between basic research and translational medicine. Several examples of ctDNA applications come from lung cancer. *EGFR* mutations were identified by droplet digital PCR in plasma drawn from NSCLC patients with superior sensitivity and specificity than sequencing results of the primary tumor sample (65). In particular, a diminished amount of mutant *EGFR* ctDNA was detected in patients with partial or complete clinical remission, whereas persistence of mutations was evident in a patient with cancer progression. Altogether, these data point to the usefulness of ctDNA analysis to monitor disease progression and to early detect treatment failure associated with acquired drug resistance. Similar results were obtained in another group of NSCLC patients, where the presence of both activating *EGFR* mutations and mutations conferring resistance to EGFR-targeted therapies were observed in ctDNA by highly sensitive single-molecule PCR (66). In another study, serial ctDNA samples obtained from NSCLC patients and subjected to whole-exome sequencing revealed an activating *EGFR* mutation at baseline and the emergence of the T790M resistance mutation upon treatment with gefitinib (61), proving the potential of ctDNA profiling to guide therapy stratification in the clinical setting. Furthermore, disappearance of *EGFR* mutations in ctDNA from NSCLC patient upon first line TKI treatment positively correlated with response rate, progression-free survival (PFS) and OS, suggesting that ctDNA monitoring during treatment could help in predicting the clinical outcome (67). Remarkably, in 2016, the U.S. Food and Drug Administration (FDA) approved ctDNA as the first liquid biopsy test to analyze the presence of specific *EGFR* mutations (exon 19 deletions or exon 21 substitution mutations) to select NSCLC patients eligible for treatment with EGFR-targeted therapy (68). The assay allows detection of mutations in plasma specimens in less than 4 hours. More recently, Schrock

and colleagues reported a large study of 1,552 patients with advanced NSCLC who were subjected to plasma DNA genomic profiling using hybrid capture-based NGS to analyze genomic alterations in 62 genes (69). ctDNA was detectable in 80% of cases, 86% of which showing at least one reportable genomic alteration (most frequently on *TP53*, *EGFR*, *KRAS*, *NF1* and *PIK3CA*) and 32% of which associated with clinically actionable targets (mostly *EGFR*). Most alterations (81%) were concordant with those identified in matched tissue samples. Once more, these results highlight the applicability of ctDNA testing in advanced NSCLC as a complementary approach to tissue testing and its particular utility at the time of progression upon targeted therapy. Notably, an expanded version of this test, covering more than 70 genes and genomic biomarkers, is now under consideration for approval by the FDA upfront the use of targeted therapies. In fact, genetic alterations associated with resistance in advanced NSCLC patients progressing to targeted therapies were analyzed in liquid biopsies by NGS (4). The most common variations were acquired mutations in *ALK* and *EGFR*, followed by amplifications. The results of this study point to the implementation of a combined ctDNA isolation + NGS strategy for patients progressing to targeted therapies in order to both clarify the mechanisms associated with acquired resistance and drive the selection of subsequent lines of treatment.

From bench to bedside: lessons from CTCs in lung cancer

CTCs have been evaluated for use as prognostic, pharmacodynamics and predictive markers to quantify MRD as well as for screening purposes, disease monitoring and therapy design (70,71). By starting directly from patient-derived material, CTCs are uniquely able to conjugate pre-clinical investigation with direct clinical evidence. With respect to lung cancer, several exciting clinical applications are being explored and developed. Currently, CTCs are detected in approximately 30–35% of patients with advanced NSCLC. However, they can be collected in larger numbers and in a wider population of patients when analyzing a higher blood volume obtained through leukapheresis (72,73). CTCs have displayed some prognostic utility in NSCLC (cut-off level of 5 CTCs/7.5 mL blood) (74) and in SCLC, where CTCs are generally more abundant than in any other disease type tested so far (range: 0–45,000 CTCs/7.5 mL blood, with a cut-off level of 50

CTCs/7.5 mL blood) (75). In most SCLC patients, the total number of CTCs is substantially reduced upon a single cycle of chemotherapy (40,75,76), in line with the high response rates observed with platinum-based treatment and in association with good prognosis (75). Lung cancer was also used as proof of concept to investigate the potential of CTCs as a liquid biopsy alternative to select personalized therapy based on the molecular characteristics of a tumor. This was exemplified in a study of *EGFR* mutation-positive NSCLC where, indeed, activating *EGFR* mutations (detected through traditional tumor biopsy analysis) were identified in 19 out of 20 patients using a microfluidic CTC enrichment platform followed by a PCR-based assay (77). Serial analysis of four patients during *EGFR*-targeted therapy revealed emergence of the T790M resistance mutation, confirming that CTCs may be applied in early identification of resistance biomarkers. One further example derives from the analysis of the *EML4-ALK* rearrangement, occurring in 3–5% of patients with NSCLC. Approximately 60% of these patients exhibit remarkable responses to *ALK* inhibition (78,79). Two independent studies have verified the applicability of *ALK*-fusion FISH testing in CTCs, showing strong correlation between the CTC *ALK*-fusion status and that of the primary tumor (80,81). Ilie and colleagues also demonstrated that both *ALK* rearrangements (revealed by FISH) and elevated *ALK* protein expression (revealed by immunocytochemistry) were detectable in CTCs originating from five patients with *ALK*-rearranged NSCLC (80). Oppositely, CTCs derived from 82 *ALK* rearrangement-negative patients did not display any positivity to FISH and immunocytochemistry testing. In the future, this type of assay could be applied as a noninvasive method to stratify patients who should receive *ALK* inhibitors, with repeated sampling during disease progression to interrogate mechanisms of resistance. In another study, Tamminga and colleagues investigated if CTCs at baseline could be considered as an indicator of worse tumor response in 86 advanced NSCLC patients treated with TKI or chemotherapy (43). Given the low CTC counts normally identified in NSCLC compared to other tumors (82), a cut-off value of just one CTC/7.5 mL blood was selected. Their results confirmed that the presence of CTCs before therapy can be considered a risk factor for worse response and survival in advanced NSCLC, irrespective of treatment (TKIs vs. chemotherapy). CTCs have shown to be prognostic for lung cancer previously (39–42) and, in general, an increase in CTC numbers during treatment is associated with worse response and lower PFS

and OS (74,83). The authors conclude that the reduced response rate in patients with detectable CTCs could be due either to epithelial to mesenchymal transition of CTC themselves, leading to increased expression of chemotherapy resistance-related genes, or to the fact that CTCs reflect a larger tumor burden or a more aggressive tumor, influencing the overall health state of a patient, causing lower drug tolerability, less responsiveness to treatment and shorter survival (43). The prognostic significance of CTC clusters and their molecular characteristics have also been investigated in SCLC (75). Interestingly, CTC clusters are rarely captured in most patients with solid tumors, but they are frequently detected in SCLC. Differently from single CTCs, clusters are non-proliferative (Ki67 expression-negative), suggesting a state of cell-cycle arrest, likely contributing to chemotherapy resistance and increased cell survival and resulting in worse prognosis.

To date, most genetic studies on CTCs have been conducted on DNA or RNA extracted from enriched CTC populations with reduced sensitivity, caused by masking of the tumor profile by wild-type DNA from leukocytes. For example, Punnoose and colleagues investigated the *EGFR* mutation status in CTCs from patients with NSCLC, revealing detectable mutations in only one out of eight patients with confirmed *EGFR* mutation in the corresponding tumor biopsy sample (41). More recently, genomic analysis of single CTCs has overcome the significant limitations imposed by leukocyte contamination, enabling the evaluation of CTC heterogeneity and often disclosing the presence of co-existing mutations within a cell. Although single-cell profiling has still to face a number of challenges related to the limited amount of starting material, the costs and complexity of the downstream processing, whole genome amplification (WGA) and NGS approaches have now shown enough robustness and reliability to be applied in CTCs as well. Nevertheless, how many CTCs are actually needed to produce a representative snapshot of each specific malignancy is still under debate (84). Ni and colleagues reported the first single-cell analysis of CTCs in patients with lung cancer (85). The authors demonstrated reproducible copy number variation (CNV) patterns between CTCs within an individual and among patients with the same histological subtype of lung cancer (NSCLC or SCLC). The authors speculate that CNV changes are a critical driver for metastasis development and that CTC-based CNV profiling could be employed as a valuable diagnostic tool. More recently, CTC single-cell sequencing has been widely applied to lung cancer.

SCLC patients with low CNV index showed significantly prolonged PFS and OS after first line chemotherapy in comparison with those with high scores, suggesting the potential of this approach for patient stratification (86). In another study, single-cell analysis of genes implicated in the metastatic process of CTCs derived from NSCLC patients revealed profound intratumor heterogeneity and uncovered predictive biomarkers for metastatic risk, proving superior accuracy in identifying patients with early-stage disease at high risk of recurrence (87). Finally, single-cell sequencing of CTCs derived from *ALK*-driven NSCLC in crizotinib-resistant patients displayed a wide variety of mutations, CNV and whole-genome duplications, highlighting heterogeneous resistance mechanisms (88). Such an approach could contribute to help clinicians in treatment personalization and selection of second line therapies.

Challenging drug resistance in lung cancer: new insights from pre-clinical studies

In *EGFR* mutant NSCLC cancer cell models, tumor heterogeneity represents a leading cause of targeted therapy failure (89-92). Moreover, sophisticated pre-clinical *in vitro* analysis have clearly shown that acquired resistance can emerge upon drug exposure, suggesting that some cells are in any case resistant to treatment (see *Table 1*). The phenomenon of drug tolerance has been extensively investigated in bacteria, where treatment of a sensitive population with an effective antibiotic often results in survival of a small fraction of dormant cells, which eventually awake and are responsible of treatment relapse (103). The same survival strategy has been also observed in NSCLC cell lines upon anti-EGFR treatments (97). In this setting, a subpopulation of cancer cells, called drug-tolerant persisters (DTPs) survives to molecular therapy by entering into a dormant state. In this condition, DTPs are characterized by a dramatic epigenetic remodeling that confers resistance to treatment by activating the pro-survival IGF1 pathway. In a pre-clinical framework, only the combination therapy based on anti-EGFR and histone deacetylase inhibitors was effective to eradicate DTPs (97). Additional studies have demonstrated that ERK1/2 reactivation occurs after few days of anti-EGFR treatments. Accordingly, the dual inhibition of EGFR and MEK was particularly effective and resulted in a durable disease control (94,95). However, even upon this combination therapy, a subpopulation of cells can survive by activating the

Table 1 Molecular resistances and innovative approaches in lung cancer

	Therapeutic strategies	<i>In vitro</i> studies	<i>In vivo</i> studies	Ref.
Mechanisms of resistance to anti-EGFR treatments				
<i>EGFR</i> ^{T790M}	Combination of EGFR inhibitors with BCL-xL and BCL-2 inhibitor	Commercial cell lines, high-complexity barcode library and patient-derived cell lines	Xenografts	(93)
<i>MET</i> amplification High levels of HGF production	Combination of EGFR inhibitor with <i>MET</i> inhibitor	Commercial cell lines	Xenografts	(89)
Reactivation of ERK signaling	Combination of EGFR inhibitor with MEK or ERK inhibitors	Commercial cell lines	GEMMs	(94)
Reactivation of ERK and AKT/ mTOR signaling	Combination of EGFR inhibitor with MEK and TORC1/2 inhibitors	Commercial cell lines	Xenografts GEMMs	(95)
High YAP/TEAD activity	Combination of EGFR inhibitor, MEK inhibitor, YAP and TEAD inhibitors	Commercial and patient-derived cell lines	Xenografts GEMMs	(96)
IGF-1R signaling and chromatin remodeling	Combination of EGFR inhibitor with IGF-1 receptor inhibitor or chromatin-modifying agents	Commercial cell lines	GEMMs	(97)
Heterogeneous alterations	Large-scale drug screening with combination of EGFR inhibitor and various inhibitors	Commercial cell line	–	(90)
<i>MET</i> amplification Transcriptional upregulation of EMT pathways	Combination of EGFR inhibitor with <i>MET</i> inhibitor	Commercial cell lines and high-complexity barcode library	–	(92)
Mechanisms of KRAS activation				
<i>KRAS</i> ^{G12C} activating mutation	Screening of various <i>KRAS</i> ^{G12C} potential inhibitors	Commercial cell lines	–	(98)
<i>KRAS</i> ^{G12C} activating mutation	Combination of <i>KRAS</i> ^{G12C} inhibitor AMG 510 with chemotherapy, targeted agents or with immune-checkpoint inhibitors	Commercial cell lines	Xenografts PDXs Patients	(99)
<i>KRAS</i> ^{G12C} activating mutation	Combination of <i>KRAS</i> ^{G12C} inhibitor MRTX849 with RTKs, MAPK/ERK, PI3K, mTOR or cell cycle inhibitors	Commercial cell lines	Xenografts PDXs Patients	(100)
<i>KRAS</i> ^{G12C} activating mutation	Combination of <i>KRAS</i> ^{G12C} inhibitor ARS-1620 with mTOR and IGF1R inhibitors	Commercial cell lines	Xenografts GEMMs	(101)
<i>KRAS</i> ^{G12C} activating mutation maintained in its active, drug-insensitive state by EGFR/AURK signaling	Combination of <i>KRAS</i> ^{G12C} inhibitor ARS1620 with EGFR, SHP2 or AURK inhibitors	Commercial cell lines	Xenografts	(102)

EMT, epithelial-mesenchymal transition; GEMMs, genetically engineered mouse models; PDXs, patient derived xenografts.

AKT/mTOR pathway (95) or entering in a senescence-like quiescent phase governed by high YAP/TEAD activity (96). Overall these results suggest that, (I) DTPs represent a critical reservoir of cancer cells that resist to long-term treatments and that may acquire *de novo* alterations and eventually emerge as fully resistant and genetically distinct populations (90,93); (II) additional options are required to increase the efficacy of molecular therapies upfront and to improve long-term patients' benefit (95,96).

The recent resolution of the KRAS^{G12C} binding pocket (98,104) has been crucial to develop mutant-directed inhibitors, able to bind covalently and trap KRAS^{G12C} in the inactive state. These pre-clinical proof of principle studies have broken down the dogma that mutant KRAS was undruggable (105,106). Indeed, two compounds have been recently explored in patients, showing promising antitumor activity in terms of objective partial response and stable disease in KRAS^{G12C}-positive adenocarcinomas (99,100). AMG 510 was tested in the first dosing cohorts and was well tolerated, thus representing a potential transformative therapy for patients harboring the KRAS^{G12C} alteration (99). In pre-clinical studies, this compound triggered a pro-inflammatory tumor microenvironment, suggesting that its efficacy is in part associated to its ability to re-establish an immune-active milieu (99). Moreover, a combination treatment based on AMG 510 and anti-PD-1 therapy resulted in an adaptive immune response inducing tumor rejection in mice also in a context where KRAS alterations were heterogeneous (99). Detailed pre-clinical studies have already conveyed that the efficacy of anti-KRAS^{G12C} therapies can be improved with the concomitant use of compounds targeting receptor tyrosine kinases, cell cycle regulators and mTOR (100,101). At the molecular level, single-cell RNA sequencing (scRNA-seq) analysis at early time points revealed diverging responses across populations of KRAS^{G12C} lung cancer cells under treatment with the mutant-directed inhibitor. Again, a fraction of cells was found stuck in a dormant state, but still able to produce new KRAS^{G12C} protein, which in turn was maintained in the active and thus drug-insensitive state (GTP-binding) by EGFR and Aurora kinase signaling (102).

Patient derived organoids (PDO): a robust platform to rapidly translate novel pre-clinical therapeutic options into clinical practice

Tumor cell lines have been extensively exploited in cancer research to identify critical pathways involved

in tumorigenesis and to pinpoint key vulnerabilities for therapeutic intervention (107). However, two-dimensional (2D) cultures suffer from different limitations, such as (I) the *in vitro* selection of specific subclones; (II) genetic drifting; (III) the lack of the original tumor architecture and microenvironment. On the other hand, patient-derived xenografts (PDXs) or xenopatient preserve the original tridimensional (3D) structure but their stabilization is extremely time-consuming and requires a large cohort of mice in drug screening. Finally, tumor growth and clonal evolution can be subjected to a negative influence by the murine microenvironment (108). The recent establishment of organotypic cultures is overcoming many of the 2D and PDX limitations. In 2009, Clevers's group demonstrated for the first time the feasibility of generating intestinal 3D cultures from *LGR5*⁺ intestinal stem cells. This *in vitro* model faithfully recapitulated the primitive intestinal architecture (109). Nowadays, tridimensional cultures, based on 3D matrix embedment, can be established from almost any tissue and tumor type (110). 3D organoids have been stabilized using different protocols from induced pluripotent stem (iPS) as well as pluripotent stem cells (PSC) of both murine and human origin (111-116). Some of the lung organotypic cultures could be expanded *in vitro* for many passages and included basal, ciliated and Clara cells. The methodology to establish short- and long-term lung cancer organotypic cultures from primary human samples has been recently reported (see *Table 2*). Histological and genomic profiling analyses confirmed that lung cancer organoids preserved both the original tumoral architecture and the primitive genetic features, thus supporting their use as a drug screening platform in personalized medicine and for biomarker validation (117-119). The superior value of organotypic cultures has been deeply investigated in gastro-intestinal and breast cancer models, where large biobanks of living organoids from both primary and metastatic lesions have been collected (111,120,121,126). Whole genome sequencing analyses have been successfully applied and high-throughput drug screening have pointed to novel promising therapeutic options (122). The new frontier in the field is now represented by the generation of co-cultures of tumor organoids with the cells of the relative microenvironment (127). This aspect is particularly relevant since diverse cellular populations of the tumor microenvironment establish complex relationships with cancer cells and influence drug response and resistance to therapy (128-130). Thus the possibility to reconstitute the tumor architecture and the

Table 2 Organotypic cultures as preclinical models

Organism	Tissue	Type	3D culture platform	Application	Ref.
3D organoid culture from PSCs					
Human	Pancreas	Normal	3D submerged culture in Matrigel	Basic research	(111)
Human	Lung	Normal	3D submerged culture in Matrigel/PLG scaffolds	Basic research	(112)
Human	Lung	Normal	3D submerged culture in Matrigel	Basic research	(115,116)
3D organoid culture from aSCs					
Mouse	Intestine	Normal	3D submerged culture in Matrigel	Basic research	(109)
3D PDO from surgical resection or biopsies					
Human	Pancreas	Primary tumor	3D submerged culture in Matrigel	Basic and translational research	(111)
Human	Lung	Primary tumor and matched normal tissue	3D submerged culture in Matrigel	Basic and translational research	(117)
Human	Lung	Primary tumor PDX tumor	3D submerged culture in Matrigel	Basic and translational research	(118)
Human	Lung	Primary tumor, metastasis, tissue from CF patients or healthy donors and lavage fluid	3D submerged culture in BME w/ or w/o Neutrophils/ALI	Basic and translational research	(119)
Human	Breast	Primary tumor and metastasis	3D submerged culture in BME	Basic and translational research	(120)
Human	Colon	Primary tumor and matched normal tissue	3D submerged culture in BME	Basic and translational research	(121)
Human	–	Metastasis from gastrointestinal tumors	3D submerged culture in Matrigel	Basic and translational research	(122)
Human, Mouse	Different tissues	Primary tumor and metastasis	3D-ALI in Collagen type I co-culture with TME	Basic and translational research	(123)
Human	Colorectal tract, Lung	Primary tumor and metastasis from different tissues	3D-ALI in Geltrex co-culture with PBL	Basic and translational research	(124)
Human, Mouse	Different tissues	Primary tumor and metastasis	Microfluidic devices with 3D spheroids in Collagen hydrogel co-culture with TME	Basic and translational research	(125)

ALI, air-liquid interface; aSC, adult stem cells; BME, basement membrane extract; CF, cystic fibrosis; PBL, peripheral blood lymphocytes; PDO, patients-derived organoids; PLG, poly(lactide-co-glycolide), PSC, pluripotent stem cells; TME, tumor microenvironment; w/, with; w/o, without.

microenvironment heterogeneity *in vitro* represents an extraordinary technical advance to design more effective therapies. In this context two methodological approaches have been explored so far. The most reliable technique consists in establishing a 3D culture which includes the tumor microenvironment and the cancer cells. Specifically, the air-liquid interface technology has been applied to establish 3D cultures of colon and lung cancer specimens along with the populations of the microenvironment. Notably, this approach allowed the preservation of the original T cell receptor heterogeneity and immune cells were maintained for 1 month in culture with the addition of specific cytokines. At the functional level, using immune checkpoint inhibitors, the authors demonstrated that the original tumor infiltrating T cells could be activated and directed against tumor cells (123). Although fascinating, this approach remains extremely risky in case of metastatic lesions, where the scarce amount of material can impact on the 3D tumor culture as well as on maintenance and growth of other populations. Alternatively, peripheral mononuclear cells can be isolated from the same patient and subsequently the T-cells can be instructed *in vitro* using the 3D PDO cultures. With this approach it is still possible to isolate and expand the tumor-directed T cells and test their tumor killing ability *in vitro*. This methodology was successfully applied to NSCLC specimens, where instructed T-cells exerted their cytotoxic activity only on tumor organoids while preserving normal lung 3D cultures (124). Finally, advances in biocompatible devices have recently resulted in the generation of novel microfluidic systems where the compartmentalization of different cells populations into separate channels can enable the setup of more sophisticated experimental designs using minimal amounts of material (131). This technology is particularly powerful to interrogate the tumor immune microenvironment, specific cell-cell interactions as well as vasculogenesis, angiogenesis, blood-brain barrier and migration/metastasis assays in a spatiotemporal setting that can mimic *in vivo* conditions more accurately (125,132-134). Overall, the possibility to integrate microfluidic devices with immune-organotypic cultures represents an extraordinary pre-clinical platform with the potential to accelerate the

translation of novel therapeutic options into the clinic.

Conclusions

In the last decade, impressive advances in both microfluidics and sequencing technologies have fuelled the debate as to which between CTCs and ctDNA will be the best alternative for pre-clinical studies and clinical use. On one side, ctDNA is appealing for its simplicity and sensitivity. Plasma can be easily collected and readily analyzed without prior enrichment and isolation procedures. These features make ctDNA the ideal option for both high throughput genotyping and monitoring response to treatment, although the information derived from it would be limited to point mutations, structural rearrangements, CNV and changes in DNA methylation. On the other side, CTCs, thank also to the rapid emergence of novel capture platforms with improved yield and simplicity, present the invaluable opportunity to study the whole cell, allowing molecular analysis at the DNA, RNA and protein level (and their reciprocal interplay) as well as biological and functional characterization, including cell morphology, immunocytochemical phenotype and presence of diagnostic epitopes. For these reasons, CTCs would be able to provide unique insights into the genetic landscape of single cells, tumor heterogeneity and clonal evolution, with significant benefits in terms of guiding personalized treatment selection. Of note, recent findings provide evidences that also tumor-educated platelets (TEPs) can be exploited as innovative diagnostic and predictive biomarkers in NSCLC (135,136). Moreover, the development of miniaturized fluidic devices to perform functional studies using CTCs as well as immune-organoid cultures opens a novel scenario where limited amounts of biological material could be sufficient to perform synthetic lethality screening also in advanced lung cancer patients. Therefore, it is likely that, in the next future, the possibility to combine genomic and functional studies using ctDNA, CTCs and microfluidic 3D cultures will be routinely explored for the isolation of predictive targets, patient stratification, real-time monitoring of disease and identification of the optimal therapeutic regimen to increase patient benefit and outcome (see *Figure 1*).

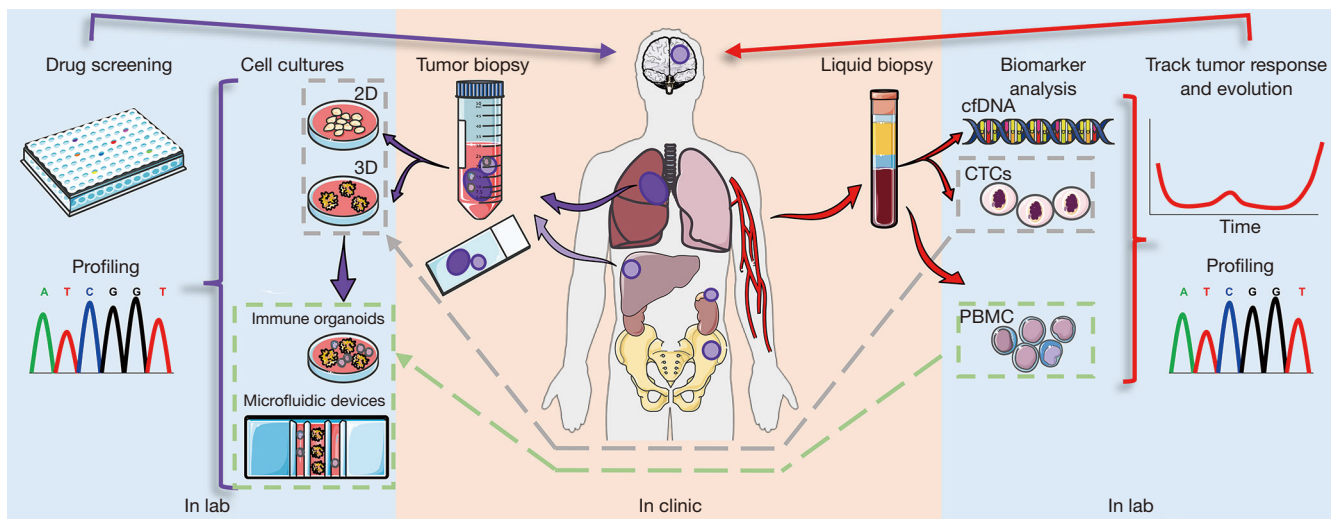


Figure 1 Moving towards pre-clinical and co-clinical molecular pipelines in lung cancer treatment. Overview of the experimental workflow: the Oncologist and Pathologist select patients and primary specimens (tumor and liquid biopsies) for molecular and functional analysis. Violet arrows indicate analyses performed on primary tumor and metastases; red arrows indicate liquid biopsy analyses. In the laboratory, upon isolation of cell free DNA (cfDNA) and circulating tumor cells (CTCs), predictive and prognostic biomarkers are isolated and validated to monitor tumor evolution and response to therapy over time. Concomitantly, Peripheral Blood Mononuclear Cells (PBMC) are used to exclude germline mutations in next generation sequencing (NGS) profiling. On the other hand, CTCs and tissue biopsies are used to establish 2D and 3D cell cultures. Tumor infiltrating lymphocytes (TIL) or PBMC are exploited to generate immune organoids as well as 3D microfluidic immune organotypic cultures. Finally, functional testing, drug screening and NGS profiling are performed on *in vitro* cultures models to identify effective pharmacological treatments and improve patients' clinical outcome. Cartoon images were obtained from <https://smart.servier.com>.

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Footnote

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