Review Article

Biology and clinical significance of circulating tumor cell subpopulations in lung cancer

Linda O'Flaherty[1,2], Harriet Wikman[2], Klaus Pantel[2]

[1]Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany; [2]Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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Correspondence to: Prof. Dr. Klaus Pantel. Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany. Email: pantel@uke.de.

Abstract: By identifying and tracking genetic changes in primary tumors and metastases, patients can be stratified for the most efficient therapeutic regimen by screening for known biomarkers. However, retrieving tissues biopsies is not always feasible due to tumor location or risk to patient. Therefore, a liquid biopsies approach offers an appealing solution to an otherwise invasive procedure. The rapid growth of the liquid biopsy field has been aided by improvements in the sensitivity and specificity of enrichment assays for isolating circulating tumor cells (CTCs) from normal surrounding blood cells. Furthermore, the identification and molecular characterization of CTCs has been shown in numerous studies to be of diagnostic and prognostic relevance in breast, prostate and colon cancer patients. Despite these advancements, and the highly metastatic nature of lung cancer, it remains a challenge to detect CTCs in advanced non-small cell lung cancer (NSCLC). It may be that loss of epithelial features, in favor of a mesenchymal phenotype, and the highly heterogeneous nature of NSCLC CTCs contribute to their evasion from current detection methods. By identifying a broader spectrum of biomarkers that could better differentiate the various NSCLC CTCs subpopulations, it may be possible to not only improve detection rates but also to shed light on which CTC clones are likely to drive metastatic initiation. Here we review the biology of CTCs and describe a number of proteins and genetic targets which could potentially be utilized for the dissemination of heterogenic subpopulations of CTCs in NSCLC.

Keywords: Circulating tumor cells (CTCs); liquid biopsy; lung cancer

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Introduction: "liquid biopsy" as blood-based biomarkers

According to the latest global report on cancer instances and mortality, lung cancer accounts for 13% of new cancer diagnoses and 19.4% of cancer-related deaths in both men and women (1). A recent study on cancer mortality rates in Europe predicts that lung cancer deaths in women will be comparable to breast cancer deaths (92,300 *vs.* 92,600, respectively) for 2017 (2). Despite improvements in drug development and treatments for non-small cell lung carcinoma (NSCLC) patients, five year survival rates remain unacceptably low at ~15%, with many patients presenting with late stage disease and secondary tumors at distant sites (such as the liver, brain, bone and contra lateral lung) at initial diagnosis. Unfortunately, lung cancer patients presenting with distant metastases have significantly reduced progression-free survival (PFS) and overall survival (OS) (3). Recently, a number of actionable genetic driver mutations and translocations have been identified for targeted therapy

for NSCLC patients. Among these gene alterations are echinoderm microtubule-associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*ALK*) fusion, epidermal growth factor receptor (*EGFR*) activating (exon 21 L585R point mutations and exon 19 deletions) and T790M mutations [confer resistance to first and second generation tyrosine kinase inhibitors (TKIs)], and ROS Proto-Oncogene 1 (*ROS1*) translocations. Furthermore, there is tremendous excitement about the potential of immunotherapy to treat NSCLC patients. For the first time, a substantial number of patients with advanced NSCLC have benefited greatly from immunotherapies, experiencing durable remissions and prolonged survival (4). Thus in recent years, checkpoint inhibitors have become an important tool for treating advanced NSCLC. The currently approved immunotherapies in NSCLC target PD-1, whereby the PD-1 inhibitors target an immune checkpoint found on T-cells. Cancer cells can use the PD 1 pathway to deactivate T cells and escape from the immune system. However, not everyone benefits from immunotherapy and a good predictive biomarker is still missing and urgently needed.

By identifying and tracking the genetic changes in primary and secondary tumors, patients can be stratified for the most efficient therapeutic regimen on the basis of screening of known biomarkers. Although solid biopsies remain the standard approach for confirming tumor histology and staging, there are limitations to their use. Namely, (I) depending on the tumor location it may not be possible to take a biopsy, (II) serial biopsy or biopsy of metastatic sites is often not feasible and (III) solid biopsies do not fully capture the heterogeneity of the tumor. A less invasive approach, involving a standard blood draw or so-called "liquid-biopsy", can facilitate multiple sampling both prior to and during treatment without risk to the patient. Importantly, genetic analysis can be carried out on liquid biopsies thus providing a 'real-time' snapshot of tumor genotype evolution and the efficacy of treatment response on a patient-to-patient basis.

"Liquid biopsy" is an umbrella term that includes, but is not limited to, molecular analysis of CTCs, circulating tumor DNA (ctDNA), tumor-derived exosomes, and circulating miRNAs. Such blood-based biomarkers may provide an important insight into tumor heterogeneity and the opportunity to investigate the biological mechanisms driving metastatic initiator cells. Therefore the benefit of a liquid biopsy over a solid biopsy is that patient screening can be carried out in a non-invasive manner to better stratify patients for therapeutic interventions and monitor patient response.

To date, a higher number of studies investigating the clinical relevance of liquid biopsies have performed analyses on CTCs or ctDNA. Although the isolation of ctDNA is less challenging and thus easier to implement in a clinical setting, analysis of CTCs offers an opportunity to investigate individual clones on a single-cell level that originate from distant tumor sites in metastatic disease. Furthermore, CTCs represent living cells with the potential of forming metastasis and thus allows for functional analyses. The presence of CTCs in the blood of a cancer patient was first observed as far back as 1869 (5) and in the past 20 years research in the field is rapidly growing to investigate the potential of CTCs in cancer diagnostic and prognostic to the clinic. As evidence of this, for lung cancer alone, there are >160 clinical trials listed on ClinicalTrials.gov which involve CTCs as investigative biomarkers. Furthermore, the successful generation of patient CTC-derived eXplant (CDX) models from SCLC (6) and NSCLC (7) patient CTCs heralded an exciting new phase of research in the field of liquid biopsy. These CDX models offer a dynamic system for better understanding drug resistance mechanisms and testing new drugs and treatment combinations.

Despite such developments, the detection and characterization of NSCLC CTCs from background noise of normal blood cells remains challenging, especially when inter- and intra-patient heterogeneity is taken into consideration. This problem could potentially be mitigated by identifying a broader spectrum of molecular markers that could be utilized for CTC enrichment. In this review we look at a number of protein and genetic markers which may contribute to the biology and clinical significance of various CTC subpopulations in lung cancer.

Biology and detection of CTCs in NSCLC

The clinical utility of CTCs is not confined to their enumeration. CTCs lend themselves well to molecular characterization such as proteomic, genomic (mutation and copy number analyses) or even mRNA and methylation analysis at a single cell level, due to the stability conferred by the whole cell structure of CTCs. Furthermore, the CTC population presents a snapshot of clonal diversity from cells originating from not only primary but also metastatic tumor sites, which cannot be accurately determined by ctDNA analysis. While ctDNA offers the advantage of not requiring a pre-enrichment step, the

specific antigens expressed on the surface of circulating tumor cells (CTCs) can also be utilized for enrichment of CTCs from the background of blood cells found in the circulation. The ongoing challenge in the ctDNA field is identifying tumor derived ctDNA from total circulating free DNA (cfDNA) which is also released into the circulation by necrotic and apoptotic healthy cells (8).

The isolation of CTCs also presents a challenge due to the very low numbers of CTCs (usually between 1–100 CTCs/7.5 mL of blood) compared to between 10^6 and 10^8 leukocytes. Thus, CTC isolation generally requires two steps: first is the enrichment of these rare cells from background blood cells and a second downstream assay, which further characterizes the enriched CTCs in a sensitive and specific manner (9). Isolation of CTCs from the whole blood can be done using the physical properties of the cell, such as size and density and also migratory properties. There are many devices and assay systems commercially available, some of which are approved as clinical diagnostics that have enabled the identification, enumeration and analysis of CTCs and nucleic acids. The most well-known device is the gold-standard, U.S. Food and Drug Administration (FDA)-cleared CellSearch® system (Janssen Diagnostics, LLC, Raritan, USA) for enumeration and isolation of CTCs from breast, castration-resistant prostate and colorectal cancers (10-12). This system identifies CTCs based on binding to anti-EpCAM ferromagnetic microbeads (enrichment) and cytokeratin (CK) and CD45 expression (detection). To date, no detection threshold has yet been defined for lung cancers for diagnostic use with the CellSearch® system. A growing number of EpCAM-independent labelling technologies detect CTCs based on their physical traits, such as size and plasticity, and negative depletion of leukocytes. Among the commercially available microfluidic platforms, Parsortix (Angle Plc, UK) and, recently, ClearCell1 (Clearbridge BioMedics Pte Ltd, Singapore), are capable of isolating CTCs in an EpCAM-independent manner based on their size and plasticity. Filtration devices, such as isolation based on size of epithelial cells (ISET) (RareCell Diagnostics, France) capture CTCs based on their size (>4 μm), excluding smaller blood cells found in the circulation. Other established methods for isolating CTCs from whole blood samples include density gradients (e.g., Ficoll) and negative depletion of CD45-expressing leukocytes using magnetic-activated cell separation (MACS) beads (Miltenyi Biotec GmbH, Germany) or the RosetteSep kit (StemCell Technologies, Canada). As this review aims to focus predominantly on the various subpopulations of CTCs in lung cancer, we will not be discussing in detail the technologies available for CTC enumeration and detection as they have been comprehensively reviewed in more depth elsewhere (9,13-21).

Heterogeneity and subpopulations of CTCs

Unlike other cancer entities, such as breast and prostate, it remains a challenge to detect CTCs in NSCLC. Interestingly, this is not the case for small-cell lung cancer (SCLC) CTCs which are more numerous and have shown a significant association with PFS and OS (22). When one considers the highly metastatic nature of NSCLC tumors, it is surprising to find so few CTCs in the circulation of NSCLC patients (15). It may be that NSCLC CTCs are present but evade detection due to the limitations of current technologies and protocols, namely the dependence of such approaches on EpCAM labelling (15).

Epithelial to mesenchymal transition and CTCs

For breast, prostate and colorectal cancers, detection of CTCs can be achieved with high sensitivity and specificity using standard epithelial markers, including cell-surface EpCAM and pan Cytokeratin markers (CK8, 18 and 19) (23).

Nevertheless, some CTCs may evade detection due to loss of epithelial marker expression, especially in NSCLC were CTCs are generally difficult to detect. In order to enter the vasculature of the circulatory system and colonize distant organs to form metastases, epithelial tumor cells need to down-regulate their epithelial properties and acquire stem-like features to enhance cell mobility and invasiveness (24). This transition is referred to as an epithelial-mesenchymal transition (EMT) and is often associated with tumor aggressiveness and resistance to therapeutic intervention (25,26). EMT is characterized by the down-regulation of epithelial markers, such as E-cadherin and EpCAM, and by the expression of mesenchymal markers, such as N-Cadherin (also referred to as CDH2 or neural-cadherin) (27) and vimentin, including transcriptional factors Snail, Zeb-1 and TWIST (28,29). In light of this, it may be likely that CTCs in NSCLC patients have more mesenchymal characteristics and thus are overlooked by EpCAM- or epithelial maker-dependent technologies.

It has been observed in NSCLC tumor tissues that an upregulation of N-cadherin and vimentin occurs along the

peripheral leading-edge compared to central tumor tissue collected from the same patient (30). From 25 NSCLC patient samples analyzed by immunohistochemical staining, Mahmood *et al.* showed a significant correlation between the expression of N-cadherin and vimentin and advanced tumor stage. Isolation of CTCs from NSCLC by ISET filtration combined with immunofluorescent imaging of mesenchymal markers (N-cadherin and vimentin) and epithelial markers (EpCAM and pan-Cytokeratin) showed that CTCs can be identified on the basis of mesenchymal marker expression (31,32). Moreover, CTCs, isolated by CD45-depletion, from 43 NSCLC patients showed an association between N-cadherin positivity and significantly shorter PFS (5 *vs.* 8 months, P=0.03, HR =2.63) (33). In this study, heterogeneous subpopulations of CTCs detected included CTCs that had the following features: N-cadherin$^+$/CK$^-$/CD45$^-$; CK$^+$/N-cadherin$^-$/CD45$^-$; CK$^+$/EpCAM$^+$/CD45$^-$ and CK$^+$/N-cadherin$^+$/CD45$^-$. Expression of N-cadherin and vimentin has also been documented in CTCs as having an association with advanced disease in breast and prostate cancer patients (34,35).

In support of the prognostic relevance of mesenchymal markers, expression of vimentin has previously been linked to poor prognosis in NSCLC (36,37). NSCLC CTCs isolated by EpCAM-independent ISET filtration showed positive expression of cell surface vimentin and higher levels of vimentin mRNA in NSCLC CTCs compared to SCLC CTCs (7). Within the same study, tumor tissues from NSCLC CTC-derived CDx models showed stronger immunohistochemical staining of vimentin than observed in SCLC CDx tumors. These findings offer further support to the hypothesis that NSCLC CTCs may be more difficult to detect than SCLC CTCs due to the mesenchymal-like features of the former. However, vimentin is not an exclusive cancer-associated marker but is also expressed by leukocytes. In light of this, vimentin expression alone may not provide a suitable basis for enriching CTCs from surrounding blood cells but, instead, may be better suited to the characterization of CTCs.

EGFR mutations status and CTCs

Approximately 15% of lung adenocarcinoma patients in Europe harbor activating EGFR mutations, with this frequency increasing to ~40% in Asia (38). On this basis, adenocarcinoma patients are routinely tested for EGFR mutations to determine their suitability for targeted therapy. Despite initial good responses to TKIs, the majority of patients relapse due to the occurrence of new resistant clones. The most common resistance mechanism is acquirement of an additional T790M point mutation in the *EGFR* gene which accounts for ~50% of cases of acquired resistance towards EGFR TKIs (39). The clinical power of assessment of the EGFR mutation status in liquid biopsy has been shown in several studies and today an FDA-approved diagnostic assay [cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Inc., CA, USA)] is commercially available for detection EGFR mutations in ctDNA.

Although detection of EGFR mutations in ctDNA is undoubtedly a powerful tool, a number of publications have shown that ctDNA and CTCs are complementary assays enabling, for example, a more comprehensive screening of the different resistance mechanisms (40,41). Sundaresan *et al.*, investigated 40 patients with EGFR-mutant tumors progressing on EGFR TKI therapy. T790M genotypes were successfully obtained in 30 (75%) tumor biopsies, 28 (70%) CTC samples, and 32 (80%) ctDNA samples. When the results from ctDNA and CTC were combined a positive result was obtained in all patients, with T790M mutations detected in an additional 35% patients in whom the concurrent biopsy was negative or indeterminate (41).

The value of CTCs as a solitary method for detecting EGFR mutations has previously been controversially discussed (42). However, the rapid technological development of reliable single cell assays have substantially increased assay sensitivity and currently enables analysis of mRNA and DNA of single cells (43-45). Accordingly, Liu *et al.*, recently performed a meta-analysis on 170 patients from eight studies with EGFR mutation data on CTCs. A high diagnostic performance of CTCs in detecting EGFR mutations was shown (AUSROC =0.99, 95% CI: 0.98–1.00) (46). Similarly, in a recent single-center study, serial sampling from 120 NSCLC patients demonstrated that activating *EGFR* mutations (L858R point mutations and exon 19 deletions) detected in CTCs had an overall 94% concordance rate with initial patient-matched solid tumor biopsies (47). For this CTC assay, CTCs were enriched from background blood cells by CD45 depletion and DNA profiling carried out by droplet digital PCR (ddPCR) with a reported specificity and sensitivity of 47% and 64%, respectively. In a pilot study a spiral microfluidic device allowing high throughput, selective picking and isolation of single CTC was used to detect T790M mutations in seven NSCLC patients. This method also showed concordant results with the biopsy sample for six samples (48). The presence of the *EGFR* point mutation, T790M, in CTCs

isolated from 27 NSCLC patients by CTC-chip was shown in another study to coincide with disease progression and reduced response to targeted therapies (17). Moreover, the authors showed a significant concordance (92%) between expression of an activating *EGFR* mutation in CTCs and patient-matched tumor tissues. Marchetti *et al.*, used the CellSearch CTC analysis platform combined with next-generation sequencing (NGS) for EGFR mutation detection. EGFR mutations were detected in 31 (84%) patients, corresponding to those present in matching tumor tissue (49). Finally, a recent publication by Park *et al.*, used a nanotechnology based CTC detection platform in NSCLC patients (50). Using this highly sensitive device the authors were able to accurately detect each patient's known mutation on single CTCs. This mRNA based method also showed that NSCLC patient CTCs heterogeneously express MET as another bypass mechanisms mediating resistance to EGFR TKIs.

Despite the still rather challenging technical requirements for sensitive mutation detection on CTCs (45), detection of *EGFR* mutations on CTCs, instead of difficult to acquire solid biopsies, can clearly facilitate the monitoring a patient's response to target therapy and identify early signs of drug resistance.

Detection of anaplastic lymphoma kinase and c-ros oncogene 1 rearrangements on CTCs

EML4-ALK fusions are found in 1–5% of NSCLC patients (51) and traditionally these rearrangements are tested in tumor biopsies to determine if a patient is a suitable candidate for targeted therapy against *ALK*. In recent developments, a growing number of studies have demonstrated that *ALK*-rearrangements can also be detected in CTCs of NSCLC patients. In an earlier study, CTCs from 18 *ALK*$^+$ and 14 *ALK*$^-$ NSCLC patients were enriched from whole blood using a combination of ISET filtration for CTC enrichment and the FDA-approved Vysis *ALK* Break Apart FISH Probe Kit (Abbott Molecular, USA) for detection of *ALK*-rearrangements (31). Using this approach, ≥4 *ALK*-rearranged CTCs per 1 mL of blood were found in all *ALK*$^+$ NSCLC patients (mean value of 11 CTCs per mL) but only one CTC with an *ALK*-rearrangement was detected in the *ALK*$^-$ NSCLC cohort. The concordance between CTCs and corresponding tumor samples was 99.9% (quantified by the κ coefficient). Interestingly, CTCs from six representative *ALK*$^+$ patients expressed the mesenchymal markers, vimentin and N-cadherin; however, expression levels of these EMT markers were heterogeneous in tumors from five of the six patients. In a subsequent study, Pailler *et al.* utilized a semi-automated microscopy approach in combination with ISET to identify ALK rearrangements with filter-adapted-FISH (FA-FISH) revealing that the number of FISH spots per *ALK*$^+$ NSCLC CTC increased with the use of optimized settings (z-stacks spaced 0.6 µm apart) during scanning (32).

NSCLC CTCs enriched using a spiral microfluidic chip and analyzed with the Vysis *ALK* Break Apart FISH Probe Kit, 25.4% of CTCs (*n*=177) were positive for ALK-rearrangements, with ALK-signal also detected in 54% of 200 cells from FFPE samples (52). Using a novel device for CTC-capture, the NanoVelcro Chip, He *et al.* demonstrated that the ALK-rearranged CTC ratio correlated to pTNM staging in ALK-positive advanced NSCLC patients (53). Within the cohort of 21 ALK$^+$ positive patients, the percentage of ALK-rearranged CTCs ranged from 19–96%, with mean percentages of 64.76±24.03%. A further study established that ALK$^+$ CTCs counts were higher in ALK$^+$ patients (n=14; 3–15 cells per 1.88 mL of blood) compared to ALK$^-$ patients (n=12; 0–2 cells per 1.88 mL of blood) (54). ALK positivity in NSCLC CTCs was recapitulated in all tumor tissue biopsies tested from the 14 ALK$^+$ patients. Interestingly, it was also reported that one patient undergoing crizotinib treatment had increasing numbers of ALK-positive CTCs corresponding to partial response and progression, thus demonstrating the potential for ALK$^+$CTC detection as a diagnostic companion. Of further interest, the successful expansion of CTCs from an ALK-positive lung adenocarcinoma patient revealed a drug resistant mutation (L1196M on the ALK gene) and responded to *in vitro* ceritinib treatment (IC_{50} of 1,664 nM) with higher efficiency than crizotinib (IC_{50} of 2,268 nM) (55). These findings highlight the possibility of utilizing ALK$^+$ CTCs for *ex vivo* drug testing and selection of effective drug treatments for ALK-positive patients.

Despite affecting only 1% of NSCLC cases, patients with *ROS1* gene fusions respond well to targeted therapy with crizotinib in clinical pilot trials (56). Pailler *et al.* isolated CTCs from four crizotinib treated patients with ROS1 rearrangements (57). Using a similar method described in their ALK study (31), *ROS1* fusions were detected in CTCs by FA-FISH and revealed heterogeneous levels of *ROS1* copy numbers in the CTCs analyzed. Interestingly, patient tumor tissues had lower *ROS1* copy numbers compared to levels detected in CTCs of corresponding patients. Moreover, a reduction in *ROS1* fusions was observed in the

CTCs of two patients who responded to treatment with crizotinib, whereas *ROS1* fusion levels remained stable in one patient non-responsive to crizotinib. Although limited by its small size, this study demonstrated the utility of CTCs in tracking patient response through analysis of biomarkers in the blood.

Programmed death ligand-1 expression on CTCs

Another notable CTC biomarker of interest includes programmed death ligand-1 (PD-L1). Advancements in immunotherapy most notably include the approval of programmed death-1 (PD-1) checkpoint inhibitors, nivolumab and pembrolizumab, for second line treatment of NSCLC patients (58,59). Just recently pembrolizumab was approved also as a first line therapy among patients with high PD-L1 expression. Although the prognostic role of PD-L1 expression on primary NSCLC tumor tissue has long been considered controversial, a recent meta-analysis clearly indicated a poor prognosis for patients with PD-L1 overexpression (60). Despite this, only few studies so far have reported the clinical relevance of PD-L1 expression on CTCs and response to immunotherapy.

In a recent study Nicolazzo *et al.* investigated PD-L1 expression on CTCs in 24 patients receiving nivolumab (61). Despite showing a significance between PD-L1 positivity and poor outcome in late stage treatment (6 months) of NSCLC patients, the authors highlighted a lack of distinction between PD-L1 positivity and outcome at baseline and early treatment (3 months) in this small patient cohort (61). Evidence for PD-L1 expression on circulating metastatic breast cancer cells (HR^+ and $HER2^-$) was also reported after analysis of $EpCAM^+$ subpopulations of CTCs using the CellSearch system (62). However, Mazel *et al.* also observed considerable inter-patient variability in the population of PD-$L1^+$ CTCs.

Recently, it has been demonstrated that PD-L1 expression is not restricted to tumor cells (63). Schehr *et al.* assessed the contribution of $CD11b^+$ myeloid cells in false positive identification of CTCs and the subsequent impact on interpretation of PD-L1 positivity in CTCs. CTCs from 19 NSCLC patients were enriched from CD45-depleted peripheral blood mononuclear cells (PBMCs) using anti-CD45 MACS beads (Miltenyi) and fluorescently stained for CD45, panCK and the myeloid marker, $CD11b^+$. By means of FACS sorting, they showed that mean expression of PD-L1 in NSCLC patient CTCs reduced substantially when $CD11b^+$ events were removed from the overall CTC population, thus highlighting the need for more rigorous exclusion markers that may otherwise contribute to the false positive identification of PD-$L1^+$ CTCs.

The role of nuclear PD-L1 (nPD-L1) expression was also reported in vimentin positive CTCs of metastatic colorectal and prostate cancer patients (64). Following enrichment by CD45 depletion and subsequent positive selection using a novel cell-surface vimentin (CSV) antibody (37), $vimentin^+$ CTCs from colon cancer patients (n=62) and prostate cancer patients (n=30) were assessed for nPD-L1 expression by immunofluorescent staining. The findings showed a significant negative impact of nPD-$L1^+$ CTCs on OS of colorectal cancer patients but no substantial effect on PFS. The inverse was, however, true for prostate cancer patients, with nPD-$L1^+$ CTCs associated with poor PFS but no effect on OS. While the robustness of PD-L1 as a prognostic biomarker for patient stratification in NSCLC requires further investigation, standardization of PD-L1 detection protocols may go some way in addressing this.

Folate Receptor alpha expression on CTCs

Another molecular target of interest is the cell-surface glycoprotein, folate receptor alpha (FRα) of the FR family (FRα, FRβ, FRγ and FRδ). Responsible for the transport of folate into the cell, FRα is the most studied isoform owing to its expression on malignant cells of epithelial origin versus negligible expression on normal cells. Importantly, with the exception of activated monocytes and CTCs, FR is not expressed on any other cells found in the circulatory system (65). In the context of primary lung cancer tissue, expression of FRα is capable of distinguishing lung adenocarcinomas (higher FRα positivity) from squamous cell carcinomas (lower FRα positivity) in an immunohistological setting (66,67). The clinical relevance of FRα was also demonstrated by an association between FRα positivity and improved OS in patients with resected adenocarcinomas (67,68). Moreover, a monoclonal antibody targeting FRα, farletuzumab, is well tolerated in a clinical setting (69) and has shown promising results, in combination with chemotherapy, in clinical trials for ovarian cancer (70) and NSCLC (71).

A recent study investigated the prevalence of FR^+ CTCs in NSCLC using a ligand-target PCR (LT-PCR) approach (71). CTCs were isolated from peripheral blood cells by CD45-depletion and anti-EpCAM magnetic beads

were subsequently used to separate CTCs into either an EpCAM$^+$ fraction or EpCAM$^-$ fraction. Interestingly, FRα was expressed on both EpCAM$^+$ and EpCAM$^-$ CTCs, with EpCAM$^-$ cells showed a higher proportion of FRα positive CTCs compared to EpCAM$^+$ cells (72). Furthermore, the presence of FRα on CTCs, using the LT-PCR detection assay, has consistently demonstrated a robust correlation with disease stage and thus may be considered to have potential clinical utility (73,74). In support of this, a recent study quantifying FR$^+$ CTCs in 162 NSCLC patients showed that 77% of the patients had ≥8.70 FR$^+$-CTC units/3 mL blood which significantly decreased following surgical resection (75). Importantly, a clear distinction was reported between individuals with lung cancer (n=197), benign lung disease (n=119) and healthy controls (n=52), thus supporting the potential prognostic relevance of FR$^+$-CTCs in NSCLC. When compared with several established clinical biomarkers, FR$^+$-CTC showed the highest diagnostic efficiency. However, as with all other potential CTC markers, not all CTCs may express FRα. Therefore, it would be advisable to include FR with a broader spectrum of circulating biomarkers in order to capture a higher proportion of the heterogeneous CTC population in NSCLC.

V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog expression on CTCs

KRAS mutations occur in ~30% of lung adenocarcinomas (76) and although there are conflicting reports on its utility as a prognostic marker, *KRAS* G12C mutations have been shown to predict lower OS in late stage NSCLC (77,78). Moreover, 9 of 38 (24%) of lung adenocarcinomas refractory to either erlotinib or gefitinib harbored KRAS mutations, further highlighting the potential of KRAS mutational status as a predictive marker for therapeutic response (79). Yet despite numerous trials, a TKI against KRAS has yet to be approved for clinical use. Guibert *et al.* could detect *KRAS* mutations in the blood of 82% of patients (n=32) by extracting DNA from ctDNA and CTCs (80). This study used a digital droplet PCR (ddPCR) approach and showed that KRAS-mutated DNA was detected in 78% (n=25/32) of cfDNA samples but that the sensitivity of this assay reduced to 34.3% (n=11/32) in CTC DNA. Challenges in detecting KRAS in CTCs has previously been reported by Freidin *et al.*, who also reported ctDNA-based assays to be more sensitive than CTC-based methods for mutant *KRAS* detection (81).

Detection of genetic alterations associated with resistance on CTCs

Apart from *EGFR* T790M mutations, other mechanisms for acquired TKI resistance include: amplification of *MET* (also known as the hepatocyte growth factor receptor alpha) (82), B-Raf proto-oncogene (*BRAF*) (83), and phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (*PIK3CA*) (84), upregulation of Erb-B2 Receptor Tyrosine Kinase 3 (HER3) and AXL Receptor Tyrosine Kinase (Axl) (85).

MET is a target of particular interest as it has been reported to correlate with clinical progression and shorter OS in lung as well as many other cancer patients (86). It is also relevant in terms of liquid biopsy as MET expression in CTC-induced bone xenografts was shown to correlate with MET expression patterns in bone metastasis in breast cancer patients (n=3) (87). In a prospective trial, Yanagita *et al.* detected *MET* amplifications in CTCs from 3 of 39 available samples (8%) in erlotinib-treated NSCLC patients with progressive disease (40). No *MET* amplifications were found in CTCs from paired baseline samples. In a recent study of NSCLC patients (n=256), 72% of ISET- and CellSearch-enriched CTCs expressed MET with 65% of patient-matched FFPE tumor sections also positive for MET expression (93% concordance) (88). Despite a significant association between CTC number and PFS, this study showed no correlation between MET expression and PFS. In a separate study using a semi-quantitative RT-PCR approach to characterize CTCs from 22 NSCLC patients, Hanssen *et al.* reported only 14.3% of cases as positive for *MET* (89). In the same study, Hanssen *et al.* also showed 85.7% positivity for *HER3*, a member of the epidermal growth factor family, in NSCLC CTCs. This is of interest as, alongside MET, HER3 overexpression is a key player in driving the mechanism of TKI resistance (90).

The frequency of *BRAF* mutations in NSCLC is relatively low (<2%) with the *BRAF* V600E mutation accounting for 50% of these and linked to reduced OS (91,92). Despite the low frequency, monitoring this mutation in the blood could identify patients who may benefit from treatment with BRAF inhibitors, such as dabrafenib, which is currently under clinical development. In a small study, Guibert *et al.* tested DNA extracted from CTCs (isolated by ISET) and cfDNA of 6 patients with lung adenocarcinomas for the *BRAF* V600E mutation.

Using ddPCR, CTCs from only one patient were found positive for *BRAF* V600E whereas the mutation was detected in the cfDNA of all 6 patients (93). However, this study was not significantly powered to draw substantive conclusions on whether CTCs are inferior to cfDNA for detecting BRAF mutations in NSCLC. Indeed, in a larger study of colorectal cancer CTCs, Kidess-Sigal *et al.* revealed a considerable concordance of 73.9% for BRAF mutations between 23 CTCs and ctDNA samples (94).

Although relatively rare [~4% of lung cancer cases (95)], the clinical importance of PIK3CA mutations in NSCLC cancer cases is evident from its role in acquired resistance to EGFR TKIs and response to PI3K inhibitors (84,96). To date, there are limited publications which describe the clinical utility of PIK3CA-positive CTCs in NSCLC. Nonetheless, detection of *PIK3CA* mutations in CTCs from 16 colorectal cancer patients showed a 77.8% correlation with the mutations found in patient-matched tumor tissues using PCR-based Sanger sequencing (94). Using a similar method, *PIK3CA* mutations in exon 9 and exon 20 were also detected by Gasch *et al.* in CTCs from HER2-negative breast cancer patients (97). In the only published study, to our knowledge, investigating PIK3CA expression in NSCLC CTCs, Hanssen *et al.* demonstrated that *PIK3CA* was expressed in 42.9% of CTCs enriched from 48 NSCLC patients (90). Moreover, detection of *PIK3CA*$^+$ CTCs corresponded to patients who were chemo-naïve or had progressive disease compared to resected and non-progressive patients with metastases.

Discussion

To date, the potential for CTCs as surrogate markers for the detection of minimal residual disease is limited due to the challenges in detecting CTCs in NSCLC. Until a reliable and robust method for detecting CTCs can be established, setting a CTCs detection threshold, which coincides with diagnostic relevance and prognostic outcome, remains to be determined. Although the frequency of CTCs detected in NSCLC using the CellSearch and EpCAM-dependent approaches remains low, a high CTC count at either baseline or during therapy has been repeatedly shown in a number of studies to negatively impact patient survival rates (42,98). However, clinically relevant results have not only been obtained for CellSearch but for various other CTC assays. Consequently, a comprehensive meta-analysis of CTCs counts from 1,576 NSCLC patients, across 20 studies, reported a significant link between poor PFS and OS and the detection of CTCs in the blood (99). Yet different technologies and detection markers report highly variable CTC counts, also when two assays are performed in paralleled, indicating differences in both sensitivities but also the existence of heterogeneous subpopulations of CTCs in NSCLC.

In this review we introduce a number of alternative biomarkers, which contribute to the heterogeneous subpopulation of circulation tumor cells and which, furthermore, could be utilized to improve the detection and isolation of NSCLC CTCs from the blood circulatory system (*Figure 1*). A number of novel therapeutic agents are either under clinical development or are already approved for clinical use for targeting the majority of the markers discussed in this review. Here we have illustrated the diverse range of studies investigating not only dysregulation of protein expression but also genetic abnormalities in CTCs which, in many cases, showed a correlation with disease progression, patient outcome or both. For most of these markers larger independent studies are still, however, needed to assess their real clinical relevance as liquid biomarkers.

By identifying and characterizing blood-based biomarkers, clinicians could track tumor progression, monitor treatment response and make informed decisions regarding the management of the patient's disease without the need for an invasive solid biopsy. Keeping in mind the considerable heterogeneity of NSCLC tumors, inclusion of a diverse range of targets for identification of CTCs in NSCLC increases the probability of detection thus providing insight into tumor burden and disease progression. Furthermore, a multi-targeted approach offers a unique opportunity to identify potential metastatic initiator cells and develop novel detection assays.

Conclusions

With the assistance of state-of-the-art technologies and the development of highly sensitive and specific assays, the identification and characterization of the dynamic subpopulations of CTCs in lung cancer, and indeed all cancer types, has the potential to deepen our understanding of the underlying mechanisms driving tumor evolution, metastases and drug resistance. By improving our understanding of these biological mechanisms we can develop novel diagnostic approaches and fundamentally improve therapeutic interventions. Importantly, the non-invasive nature of liquid biopsies allows for sequential

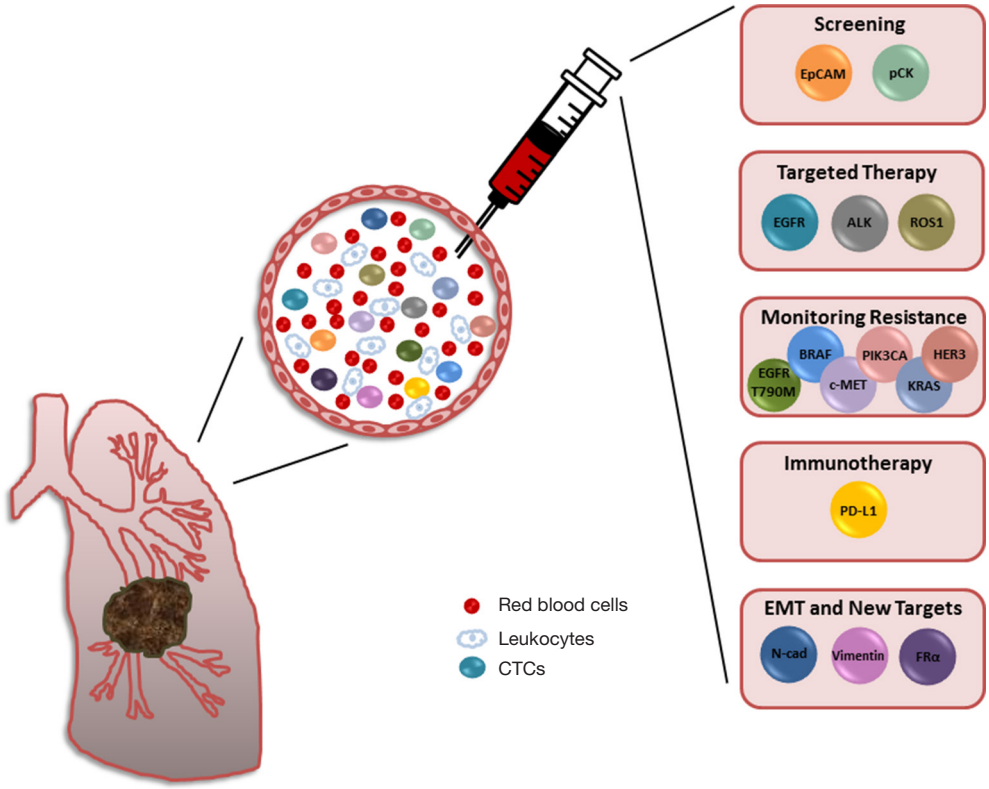

Figure 1 Identification and characterization of diverse CTC subpopulations in NSCLC could lead to a better understanding of the dynamic molecular mechanisms driving metastatic initiation and drug resistance, thus improving patient stratification and outcome. CTC, circulating tumor cell; NSCLC, non-small cell lung cancer.

sampling, with low risk to the patient, which is conducive to: 'real-time' monitoring of therapeutic response, early detection of resistance markers, prognosis and prediction of patient outcome. With such growing evidence in support of the clinical utility of liquid biopsies, the prognostic value of CTCs as surrogate biomarkers of disease progression cannot be underestimated.

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Footnote

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