Chapter

Liquid Biopsy Analysis of Circulating Tumor Biomarkers in Lung Cancer

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Abstract

Risk stratification, prognostication and longitudinal monitoring of therapeutic efficacy in lung cancer patients remains highly challenging. It is imperative to establish robust surrogate biomarkers for identifying eligible patients, predicting and effectively monitoring clinical response as well as timely detecting emerging resistance to therapeutic regimens. Circulating tumor biomarkers, analyzed by liquid biopsy, are primarily composed of nucleic acid-based circulating tumor DNA (ctDNA) and an aneuploid cell-based category of circulating tumor cells (CTCs) and circulating tumor-derived endothelial cells (CTECs). Unlike ctDNA, cancer cells are the origin of all categories of various tumor biomarkers. Involvement of aneuploid CTCs and CTECs in tumorigenesis, neoangiogenesis, tumor progression, cancer metastasis and post-therapeutic recurrence has been substantially investigated. Both CTCs and CTECs possessing an active interplay and crosstalk constitute a unique category of cellular circulating tumor biomarkers. These cells concurrently harbor the intact cancer-related genetic signatures and full tumor marker expression profiles in sync with disease progression and therapeutic process. Recent progress in clinical implementation of non-invasive liquid biopsy has made it feasible to frequently carry out ctDNA analysis and unbiased detection of a full spectrum of non-hematologic circulating rare cells including CTCs and CTECs in lung cancer patients, regardless of variation in heterogeneous cell size and cancer cell surface anchor protein expression. *In situ* phenotypic and karyotypic comprehensive characterization of aneuploid CTCs and CTECs, in combination with single cell-based genotyping and improved ctDNA analyses, will facilitate and benefit multidisciplinary management of lung cancer.

Keywords: CTC, CTEC, ctDNA, therapeutic resistance, aneuploidy, iFISH

1. Introduction

Recent progress in multidisciplinary management of advanced lung cancer has triggered enthusiasm in investigating both prognostic roles of tumor microenvironment (TME) and clinical utilities of liquid biopsy in lung cancer patients [1, 2]. How the tumor-reprogrammed lung TME promotes primary tumor progression and cancer metastasis remains to be further elucidated [1].

Aberrant stromal and infiltrated immune cells, sustained neovascularization, as well as dysfunctional neoangiogenic vasculatures in solid tumors all contribute

towards constituting an immunosuppressive TME suitable for cancer cell growth and metastasis [3]. Tumor-derived endothelial cells (TECs) participate in making up the lining of neoangiogenic vasculatures in the TME and accelerating tumor progression [4, 5]. Following their shedding into peripheral blood, $CD31^-$ cancer cells and $CD31^+$ TECs turn into circulating tumor cells (CTCs) [6] and circulating tumor-derived endothelial cells (CTECs) [7, 8], respectively. Beyond peripheral blood, tumor cells and TECs may also disseminate into body fluid including bone marrow (BM), malignant pleural effusion (MPE), ascites and cerebrospinal fluid (CSF), etc. These cells are respectively termed as disseminated tumor cells (DTCs) [9] and disseminated tumor-derived endothelial cells (DTECs). The non-hematologic circulating rare cells, consisting of CTCs, CTECs, DTCs and DTECs, possess the malignancy hallmark of aneuploidy [10–14] and play a fundamental role in tumorigenesis, neoangiogenesis, tumor progression, cancer metastasis and relapse [7, 13].

Liquid biopsy provides applicable and convenient choices for analyzing tumor-derived cells and molecules in cancer patients' circulation system [15], which is particularly adequate for lung cancer as it does not require an invasive and harmful procedure to perform a conventional pathological biopsy on the malignant lesion in lung. Liquid biopsy utilizes non-invasive approaches to reveal the molecular landscape of neoplasm in real time and facilitate management of lung cancer throughout treatment process, from identifying eligible patients, dynamically monitoring therapeutic efficacy to detecting minimal residual disease and emerging resistance [16] with respect to guiding personalized precision therapy [2].

Being as a category of liquid biopsy technologies, analysis of tumor cell genome-derived circulating tumor DNA (ctDNA) has been applied to assist management of advanced stage lung cancer [2, 17]. The cell-free biomarker ctDNA measurements show rapid response to administration of therapeutic agents. Recent advance in molecular genotyping in terms of identifying genetic mutations in ctDNA has successfully guided therapies targeting mutant *EGFR* or the *EML4-ALK* rearrangement in lung cancer patients [18, 19]. However, the specificity and sensitivity of ctDNA assay remain challenging [20, 21]. Compared to ctDNA, CTC has presented its unique advantage in terms of being as an effective response measure of prolonged survival for metastatic cancer patients in multiple clinical studies [22]. It has been realized that aneuploid circulating rare cells constitute a unique category of viable cell-based cellular circulating tumor biomarkers. Those cellular circulating tumor biomarkers contain intact genetic signatures and full protein expression profiles along with tumor progression and throughout clinical treatment process [7, 13]. The clinical relevance of aneuploid circulating rare cells in the context of tumor angiogenesis [23], cancer metastases and prognosis [6, 9] was described elsewhere [9, 24]. Detection of CTCs and CTECs has been clinically applied to prognosticate lung cancer patients [22], evaluate or monitor therapeutic efficacy in both cancer patients [25–27] and patient- or CTC-derived xenograft tumor mouse model (PDX, CDX) [28–31]. Moreover, examination of CTCs and CTECs has been successfully utilized to timely detect emerging therapeutic resistance [32–36] as well as postsurgical cancer relapse [37, 38]. Overall, availability of analysis of circulating rare cells has brought extraordinary depth by allowing feasible frequent examination of the whole intact target cells and their molecular contents including cancer-related DNA, RNA and tumor marker proteins [21]. Other liquid biopsy-relevant genotyping strategies conducted on circulating exosomes, microRNA, mRNA, metabolites and tumor-educated platelets are immature and remain to be further optimized and clinically validated [2, 17].

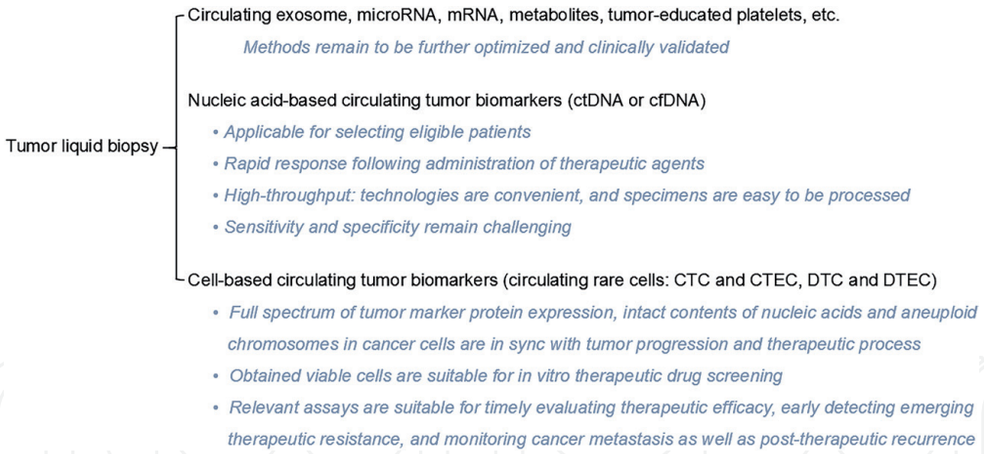

Figure 1.
Categorization of tumor liquid biopsy. Various cellular and molecular approaches are applied in the non-invasive tumor liquid biopsy to detect nucleic acid-based and cell-based circulating tumor biomarkers.

Categorization and clinical utilities of tumor liquid biopsy, primarily composed of nucleic acid-based and cell-based circulating tumor biomarker analyses, are depicted in **Figure 1**.

2. Hypoxic tumor microenvironment in lung cancer

The lung TME is a complex, dynamic system comprised of tangled interactions among carcinoma cells and their surrounding cells in a hypoxic environment [39, 40]. Aside from non-cellular compositions of cytokine and extracellular matrix, the cellular components of the lung cancer TME consist of undifferentiated cancer stem cells (CSCs) [41] and their differentiated progeny tumor cells possessing either intrinsic or induced plasticity [42]. In addition, a variety of cells other than neoplastic cells also localize in the TME, which, able to foster both tumor growth and dissemination, are composed of stromal cells and non-stromal immune cells. Tumor-associated stromal cells consist of cancer-associated fibroblasts (CAFs), pericytes, adipocytes and endothelial cells (ECs) that make up the lining of tumor vasculature. The innate immune cells in the TME encompass dendritic cells, monocytes, macrophages and lymphocytes. The major components of lymphocytes in the TME are tumor-infiltrating T cells which are recognized as a hallmark of cancer [43]. Among different subtypes of T cells in the lung TME, $CD3^+/CD8^+$ cytotoxic T cells and $CD3^+/CD4^+/CD25^+$ regulatory T cells (Tregs) are the most representative subpopulations. Cytotoxic T cells exhibit anti-tumor activity which is negatively regulated by the $FOXP3^+$ immune-suppressive Tregs [44]. Alike prognostic factors of immune cells in the lung TME, the $FOXP3^+$ Tregs correlate with poor prognosis [44] and early recurrence, particularly in node-negative NSCLC patients [45].

Hypoxia, a common phenomenon in malignant neoplasm, leads to acquisition of epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndoMT) phenotypic plasticity by epithelial cancer cells and endothelial cells, respectively. Hypoxia-inducible factor (HIF) pathway is the most distinctive intracellular signaling event that triggers and regulates EMT and EndoMT [7]. HIF pathway is activated in the hypoxic lung TME, resulting in nuclear translocation of HIF-1α and subsequent heterodimerization with HIF-1β in the nucleus [46]. HIF-1α/β heterodimers subsequently interact with NFκB to promote a series of downstream signaling cascades. Hypoxia is, therefore, the vital inducer of EMT and

EndoMT [47, 48] which fundamentally constitute the intracellular central hub of tumor neovascularization and cancer metastasis [7].

In the hypoxic TME, active crosstalk among carcinoma cells and their associated stromal cells accelerates lung cancer development by promoting tumor expansion, invasion and disease progression [49, 50]. The lung hypoxic TME thereby significantly impacts both malignant tumor progression and treatment response. Impaired vascularity and hypoxia will lead to an increased metastasis potential and treatment resistance in lung cancer [39].

3. ctDNA

ctDNA is released from apoptotic or necrotic cancer cells either in the TME of primary/metastatic lesions or in peripheral circulation. ctDNA levels correlate with tumor burden and response to therapy in NSCLC patients [51, 52]. In contrast to normal cells, neoplastic cells possess tumor-specific somatic alternations in the genome. Mutations harbored by ctDNA, including both point mutations and structural alternations (such as genome-wide copy number variations and rearrangements), correspond to that in primary tumors [21].

3.1 Clinical application of ctDNA

Following rapid evolvement of PCR and next generation sequencing (NGS)-based ctDNA analysis, its clinical application as a high-throughput diagnostic test has been facilitated in several areas. **(i)** Early detection of lung cancer: localized lung cancer at early stage sheds DNA into peripheral circulation and detection of methylated ctDNA may help diagnose early stage lung cancer [53]. **(ii)** Tumor genotyping to identify lung cancer patients eligible for mutation-targeted therapies: the most representative example is to examine sensitizing exon 19 deletions and the L858R mutation as well as the resistance mutation T790M in plasma ctDNA. All will guide administration of EGFR-Tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib and afatinib to lung cancer patients [18]. **(iii)** Surrogates of therapeutic efficacy: ctDNA dynamics is able to predict benefit of immunotherapy [54] and may also correlate with chemoradiation efficacy in lung cancer patients [55]. **(iv)** Identification of localized lung cancer at high risk of disease relapse: compared to conventional histopathological criteria in identifying post-therapeutic localized lung cancer patients suitable for personalized adjuvant therapeutic setting, cancer personalized profiling by deep sequencing (CAPP-seq) ctDNA analysis is able to detect post-surgical minimal or molecular residual disease (MRD), thereby identifying patients bearing the lowest disease burden eligible for adjuvant therapy [21, 56]. **(v)** Early detection of lung cancer relapse: whole genome analysis of ctDNA may directly identify tumor-derived structural alternations comprised of chromosomal copy number changes and rearrangements, including specific amplification of cancer driver genes (*ERBB2*, *CDK6*, etc.) that correlate with cancer recurrence [57]. In addition, phylogenetic ctDNA profiling was also reported to enable detection of recurrent NSCLC at early stage [58].

3.2 Limitations and improvement of ctDNA analysis

Advances in next generation DNA sequencing technologies have promoted clinical application of ctDNA as a tool to facilitate management of lung cancer, such as earlier detection and improvement of therapeutic outcomes by enabling early intervention, etc. However, limitations of ctDNA have recently attracted

increasing attention. Cancer-related genetic contents carried by fractured ctDNA is limited due to its 90–150 base pairs of small fragments [2]. Moreover, in carcinoma patients, little amount of cancer-related ctDNA co-exist with much larger amount of cancer-irrelevant cell free DNA (cfDNA) shed from normal cells in peripheral blood [2, 17]. This raises notable concerns regarding the specificity and sensitivity of ctDNA analysis [20, 21], particularly for low-frequency mutation detection in early stage NSCLC patients [59]. Such concern has been recently further reenforced by copious data analyses co-performed by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), indicating that clinical utility and validity of ctDNA in early-stage cancer detection, treatment monitoring, or residual disease detection in a variety of cancer patient are still vague and inconclusive [60]. Extensive clinical studies, performed by the improved technologies with higher sensitivity and specificity, will further validate and reveal clinical utilities of ctDNA.

4. Aneuploid CTCs and CTECs

Almost all different types of tumor biomarkers originate from neoplastic cells. CTCs and CTECs dynamically comprise integral molecular landscape of both cancer-related genetic variations and tumor marker expression along with tumor progression and clinical treatment process.

4.1 Aneuploidy in malignant cancer cells

Aneuploidy refers to either a gain or loss of chromosomes in a cell. Unlike constitutional aneuploidy, somatic aneuploidy is the most common feature of human carcinomas [11, 12]. In particular, aneuploid chromosome 8 (Chr 8) was observed in neoplastic cells of almost all solid tumors, including lung cancer [61].

Aneuploidy is a cellular transformation-related dynamic chromosome mutation event regulated by cell fusion and a number of mitotic genes [7, 62]. Mutations of those mitotic genes were identified in cancer cells, implicating such mutation in induction of mis-chromosome segregated aneuploidy in neoplastic cells [63]. Aberrant ploidy of extra chromosomes in cancer cells was found to relevant to genomic instability [64]. In addition, gain or deletion of hundreds of genes brought by aneuploidy in carcinoma cells results in profound varieties of phenotypes, which further drives cancer development, evolution, heterogeneity, lethal progression, drug resistance and therapy failure [14, 65]. Of extreme importance was the discovery that the degree of aneuploidy is proportional to the grade of malignancy and genetic instability of neoplasm [66, 67], showing that the higher the degree of aneuploidy, the higher the frequency of *KRAS* and *TP53* mutations, and the higher the malignancy grade of cancer cells [62, 67, 68].

4.2 Cytogenetic abnormalities in CTCs and CTECs

In the lung cancer TME, alike aneuploid neoplastic cells, a majority of ECs in tumor vasculature are aneuploid TECs [69] that could be derived from either endothelialization of malignant lung cancer cells or cancerization of stromal ECs [7, 70]. Abnormal neovasculature composed of TECs possesses loosened junctions between ECs, resulting in an increased vascular permeability and transendothelial intravasation as well as extravasation during tumor metastasis. Aneuploid TECs, harboring dual-properties of endothelial vascularization ability and cancerous malignancy [71], were reported to contribute to tumor progression [5]. Following shedding into

blood, both CTCs and CTECs adopt molecular properties from their parental cells in the TME of primary lesion, including cytogenetic abnormalities of aneuploidy. Each subcategory of those aneuploid circulating rare cells correlate with distinct clinical endpoints, such as targeted distant cancer metastasis [72, 73] and resistance to chemo- [33, 34] or immunotherapy [36].

5. Co-detection, comprehensive characterization and clinical value of diverse subtypes of lung cancer CTCs and CTECs

5.1 Conventional strategies to detect lung cancer CTCs

Several strategies were applied to attempt to detect CTCs in lung cancer patients [74]. CTC surface anchor protein (such as CD326 EpCAM)-dependent isolation (e.g. CellSearch) and cell size-exclusion filtration to enrich large cell size CTCs (>WBC size) are the most representative conventional approaches. However, it has been realized that clinically relevant small cell size CTCs (≤WBC size), such as mesenchymal CTCs [75], are lost throughout the filtered depletion of WBCs, raising non-negligible concerns with respect to specificity and sensitivity for cell filtration strategy [76, 77], particularly for lung cancer CTC detection [78]. CellSearch technology relies on positive expression of EpCAM and cytokeratin (CK) for isolation and identification, respectively. This method, restricted to both EpCAM and CK double-positive cells, is able to effectively detect CTCs shed from some particular types of solid tumors expressing abundant epithelial marker EpCAM, such as colon, breast and prostate cancers [79]. However, a majority of CTCs in various carcinoma patients exhibit a highly dynamic distribution of EpCAM during cancer progression and metastasis [80, 81]. Additionally, expression of EpCAM and CK is down-regulated during EMT in the process of CTC formation [81, 82]. Furthermore, most lung cancer CTCs exhibit either low or non-expression of EpCAM [83, 84]. Those inherited cell biological "hurdles" inevitably lead to a false negative detection of the "uncapturable" and/or "invisible" lung CTCs by the conventional approach [85]. It is therefore necessary to develop an alternative strategy, beyond restriction to EpCAM and CK double positive expression, to effectively isolate, identify, comprehensively characterize and classify a variety of highly heterogeneous aneuploid circulating rare cells in lung cancer patients.

5.2 *In situ* phenotypic and karyotypic characterization of aneuploid CTCs and CTECs by iFISH

Aside from respectively addressing nucleic acid, tumor marker proteins, or cell morphology alone, a comprehensive strategy integrating subtraction enrichment (SE) and immunostaining-fluorescence *in situ* hybridization (SE-iFISH) has been developed to effectively enrich and identify heterogeneously sized circulating rare cells [8, 61]. Following non-hypotonic removal of RBCs, subtraction enrichment is able to effectively enrich circulating rare cells in varieties of cancer patients including NSCLC and small cell lung cancer (SCLC) [86], regardless of cell size variation and the target cell surface anchor protein expression. Following efficient enrichment, iFISH co-detects tumor marker expression and chromosome aneuploidy in enriched non-hematologic circulating rare cells (CRCs) [61]. Besides, iFISH is also able to detect aneuploid hematologic rare cells derived from lymphoma and myeloma ($CD45^+$, aneuploid in Chr 12). As depicted in **Figure 2**, the most representative populations of the primary entity of non-hematologic aneuploid circulating rare cells, identified by iFISH, are CTCs/CTECs in peripheral blood and DTCs/DTECs in

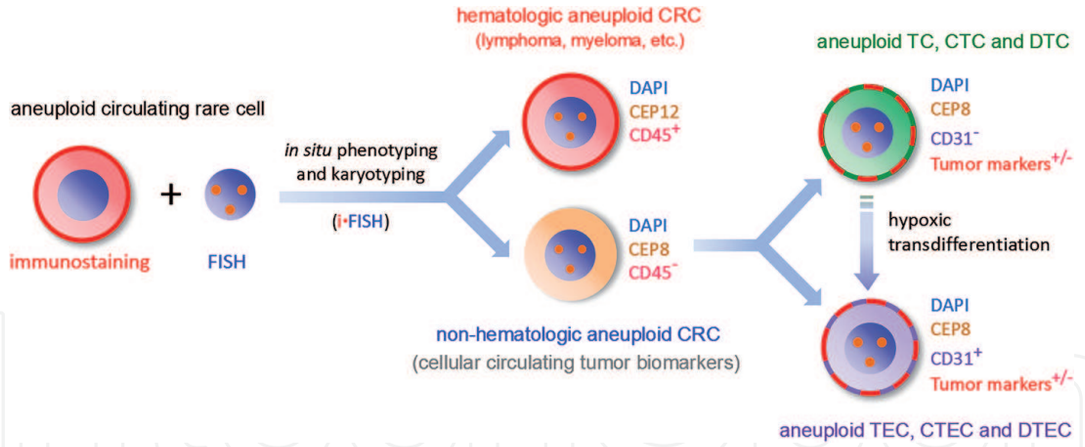

Figure 2.
Categorization of aneuploid circulating rare cells comprehensively identified and characterized by iFISH. Aneuploid circulating rare cells (CRCs) are classified into hematologic and non-hematologic categories. The former class is composed of lymphoma, myeloma, etc. which are aneuploid in chromosome 12. The non-hematologic category consists of CD31⁻ CTCs and CD31⁺ CTECs in blood, DTCs as well as disseminated TECs (DTECs) in body fluid (bone marrow, malignant pleural effusion, ascites, cerebrospinal fluid, etc.). The aneuploid non-hematologic CRCs, either expressing tumor markers or not, constitute a unique category of cell-based cellular circulating tumor biomarkers. In the hypoxic environment, some CD31⁻ tumor cells (TCs) may transdifferentiate into CD31⁺ TECs either in vivo *or* in vitro.

body fluid. Under hypoxic conditions, some CD31⁻ tumor cells (TCs) could transdifferentiate into CD31⁺ TECs, both *in vivo* and *in vitro* [7, 87, 88].

Based upon the degree of aneuploidy, tumor marker protein expression and cell morphology (large, small, cluster or microemboli), each category of circulating rare cells can be classified into diverse subtypes. Each subtype of cells respectively possesses distinct clinical values.

5.3 Clinical significance of CTCs and CTECs in lung cancer

5.3.1 Quantification

Clinical utilities of detecting CTCs and CTECs in management of NSCLC and SCLC have been investigated along the axis of "early diagnosis–treatment–relapse" [89]. Though low-dose CT (LDCT) screening was reported to reduce lung cancer mortality in low-risk populations [90, 91], its extensive application is limited due to relatively low sensitivity in high-risk populations [17, 90], unavailability of frequent re-examinations within a short period as well as socio-economic affordability. As a diagnostic marker of lung cancer, non-invasive and periodic detection of CTCs and CTECs may provide a compensatory choice to allow an effective early diagnosis of lung cancer [17, 92–94]. Multiple studies indicated that lung cancer CTCs could be detected several months prior to radiographic appearance of the primary lesion [74, 95].

With respect to diagnosed lung cancer patients, the quantity of CTCs was found to correlate with patients' pathological staging as well as amount of cytokeratin 19-derived Cyfra 21–1 in plasma [86, 96]. CTC is a risk stratification parameter for NSCLC in terms of distant metastasis [73, 97]. Prognostic values of CTCs in therapeutic lung cancer patients were published elsewhere [86, 98], indicating that baseline lung CTC counts were associated with patients' poor prognosis and response to treatment [99]. Compared to evaluation of therapeutic efficacy performed by CT scanning and RECIST criteria, quantitative change in CTCs occurs ahead of conventional medical imaging examination [86, 100], suggesting that cellular response to therapeutic regimens is more sensitive than observable size variation in imaged tumor mass.

Close attention has been recently focused on whether surgical resection may promote a quantitative increase in lung cancer CTCs. Although a study performed by the EpCAM-dependent strategy indicated that surgical approaches did not impact CTC quantity [101], the conclusion was uncertain due to the reality that the applied technology was biased in restricting to CK and EpCAM double-positive CTCs which account for only a very small proportion of overall lung CTCs. Nonetheless, several studies performed by others using different technical platforms indicated that surgical manipulation indeed increased CTC quantity either in pulmonary venous (PV) blood during surgery [102, 103] or in post-surgical patients' peripheral blood [104]. Increased CTCs in PV were reported to associate with patients' poor prognosis [103, 105]. Similar to association of post-surgical hepatocellular carcinoma (HCC) CTCs with cancer relapse [38], detection of CTCs in PV during operation or in post-surgical peripheral blood also enables early detection of lung cancer recurrence, particularly in the post-resected lung cancer patients [37, 106–108].

5.3.2 Molecular characterization

In addition to enumerating cell number alone, molecular characterization of DNA, RNA, chromosomes and proteins in circulating rare cells has been carried out to investigate the clinical relevance of molecular landscape in diverse subcategories of lung CTCs and CTECs [36, 93, 109].

Tumor-associated DNA copy number aberrations (CNAs) profiling illustrated distinctive genetic features in chemosensitive and chemorefractory SCLC CTCs [110], that will be beneficial to patients' personalized precision therapy. Besides DNA, the quantity of several tumor markers' mRNA, such as CEA mRNA in both pre- and post-surgical NSCLC patients, may serve as an independent prognosticator for poor prognosis [111].

Compared to a significant reduction in risk of mortality in post-surgical NSCLC patients who had near-diploid tumors [68, 112], subjects possessing aneuploidy in lung cancer cells exhibited a significant increase in risk of death [112]. Recent studies demonstrated that aneuploidy plays a critical role in chemoresistance in gastric cancer patients [33, 34] as well as in metastatic "patient-derived xenograft tumor mouse models" (mPDX) exhibiting primary gastric cancer metastasizing to lung [30]. For instance, gastric CTCs with trisomy 8 were found to possess intrinsic chemoresistance, whereas multiploid (≥pentasomy 8) CTCs displayed acquired resistance to cisplatin. It is logical to speculate that aneuploid lung cancer CTCs may share the similar property of aneuploidy-related therapeutic resistance.

Efficient identification of lung cancer patients eligible for targeted therapies remains a challenging topic. Compared to conventional detection of *ALK* rearrangement on biopsy specimen with respect to identifying subjects for crizotinib treatment, detection performed on CTCs to examine *ALK* rearrangement provides a better alternative in terms of rapidity and repeatability [13, 113, 114]. Targeted therapy on epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (gefitinib and erlotinib TKIs) has been profoundly applied to eligible NSCLC patients. Single cell-based analysis of genetic abnormalities, such as exon 19 deletion/*EGFR* L858R TKI-sensitizing mutation [115] and T790M TKI-resistance mutation in CTCs, could serves as an adequate alternative to identify eligible patients [116] and timely monitor emerging acquired therapeutic resistance to TKIs throughout therapy [117, 118]. Interestingly, compared to the 33% positive detection rate of *EGFR* L858R in ctDNA, 92% of the same cohort showed such mutation in their CTCs [117].

Next-generation sequencing (NGS) successfully guided *in vitro* drug screenings carried out on the cultured primary CTCs [119]. One study demonstrated that NGS performed on the cultured metastatic tumor cells which were enriched from cerebrospinal fluid in breast cancer patients led pinpointing of chemotherapeutic agent palbociclib (the synthetic CDK4/6 inhibitor) upon identifying single nucleotide variant (SNV) in those cells [120]. A similar *in vitro* therapeutic drug screening strategy performed on 3D cultured CTCs was reported to help direct potent lung cancer precision therapy [121]. In addition to analyzing DNA mutations in pooled CTCs, the single-cell based DNA [29] or RNA sequencing [31] performed on chemosensitive and chemoresistant CTC-derived xenografts (CDX) demonstrated that intratumoral heterogeneity (ITH), which was constituted of coexisting subpopulations of cancer cells with heterogeneous gene expression, led to the development of platinum-resistance in SCLC patients [29, 31]. A similar study performed by others on SCLC PDX and CDX confirmed that these models were able to capture the mutational landscape and functional traits from their primary donor tumors [28].

Aside from genetic and karyotypic characterization, phenotypic analysis of tumor marker protein expression in CTCs provides additional prognosticating value. For instance, EpCAM and Vimentin are the epithelial and mesenchymal markers of EMT and EndoMT, respectively [122, 123], both showing particular clinical outcomes in carcinoma patients. EpCAM$^+$ CTCs and DTCs are able to lead oligometastasis to lung in breast carcinoma patients [72]. Moreover, CTCs expressing EpCAM correlate with poor prognosis in lung cancer patients [84]. Vimentin$^+$ CTC is another independent prognosticator for poor prognosis and survival. Positive detection of Vimentin$^+$ CTCs at baseline has been recently reported to associate with lung cancer's hepatic metastasis and patients' poor prognosis [73].

5.3.3 Clinical utilities of co-detection of CTCs and CTECs

Most efforts made on liquid biopsy have, so far, been primarily focusing on CTCs. In comparison with CTCs, the aneuploid CD31$^+$ CTECs, harboring mixed properties of epithelium, endothelium, mesenchyme, aneuploidy, malignancy and mobility, are expected to perform an important role in tumorigenesis, progression, metastasis and neovascularization [7]. Since the existence of CTECs in cancer patients was reported for the first time [8], clinical values of CTECs in a variety of carcinoma patients have been illustrated [7, 36, 93, 124]. Compared to CTCs, lung cancer CTECs appear to be more relevant to therapeutic resistance and disease progression. Particularly, in NSCLC patients subjected to the checkpoint blockade immunotherapy (nivolumab), unlike nivolumab-sensitive PD-L1$^+$ CTCs which revealed a quantitative decrease following treatment, the number of post-immunotherapeutic aneuploid PD-L1$^+$ CTECs increased. Patients possessing post-immunotherapeutic aneuploid PD-L1$^+$ CTECs showed a significantly shorter PFS compared to those without PD-L1$^+$ CTECs [36]. Innovative attempts to therapeutically target CTEC-relevant EndoMT and aneuploidy will vitally impact aneuploid CTECs and ultimately improve lung cancer patients' treatment efficacy [125, 126]. As a novel and mobile therapeutic target, elimination of CTECs in cancer patients is expected to promote an effective obstruction in cancer metastasis.

Detection and clinical values of advanced molecular characterization of lung cancer CTCs and CTECs are summarized in **Table 1**.

To maximize clinical values of CTCs and CTECs, it is ideal to co-characterize all three elements of nucleic acids, tumor marker protein expression and cellular morphology in target cells. Such three-in-one comprehensive co-detection and molecular characterization of aneuploid circulating rare cells will effectively and

Clinical values of lung cancer CTCs and CTECs	References
Early diagnosis	[17, 74, 92, 93, 95]
Pathological staging	[86, 96]
Identification of eligible patients (TKIs, *ALK* crizotinib)	[13, 114, 116]
In vitro therapeutic drug screening	[120, 121]
Under treatment	
Risk assessment for distant metastasis	[73, 97]
Prognosis	[72, 73, 84, 86, 98–100, 112]
Post-surgery (prognostic value)	[102–105]
Timely monitoring therapeutic resistance	[31, 36, 110, 117, 118]
Early detection of recurrence	[37, 74, 106–108]
Advanced molecular characterization	
Aneuploidy and *ALK* rearrangement	[13, 112–114]
Co-detection of aneuploidy and tumor marker expression in CTCs and CTECs (iFISH)	[8, 36, 61]
CTC-derived xenograft (CDX)	[28, 29, 31]
Single cell-based DNA sequencing	[28, 29, 110, 116, 118]
Single cell-based RNA sequencing	[31]

Table 1.
Clinical utilities of detecting lung cancer CTCs and CTECs.

efficiently assist modern multidisciplinary management of lung cancer with respect to early-stage screening, identification of eligible patients, selection and optimization of therapeutic regimen, risk stratification, minimal residual disease detection, timely evaluation of therapeutic efficacy, monitoring treatment resistance and early detection of post-therapeutic recurrence.

6. Conclusions

Both aneuploid $CD31^+$ CTECs and $CD31^-$ CTCs compose a unique pair of cellular circulating tumor biomarkers that have an active crosstalk and interplay in circulation, thus promoting lymphogenous and hematogenous cancer metastasis as well as disease progression. CTECs, bearing properties of malignancy, vascularization and mobility, serve as a significant, versatile player in tumor neovascularization and cancer metastasis. Clinical implementation of advanced co-detection and comprehensive characterization of all diverse subtypes of aneuploid CTCs and CTECs, in combination with single cell-based genetic signature profiling and improved ctDNA analysis will help improve and profit current and future cancer research and precision management of patients with a variety of carcinomas, including, but not limited to, lung cancer.

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Conflict of interest

i•FISH® is the registered trademark of Cytelligen. Dr. Peter P. Lin is the president at Cytelligen. No additional COI to be disclosed.

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