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CANCER INNOVATION

REVIEW

Advances in the role of circulating tumor cell heterogeneity in metastatic small cell lung cancer

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Abstract

Small cell lung cancer (SCLC), a highly aggressive malignancy, is rapidly at an extensive stage once diagnosed and is one of the leading causes of death from malignancy. In the past decade, the treatment of SCLC has largely remained unchanged, and chemotherapy remains the cornerstone of SCLC treatment. The therapeutic value of adding immune checkpoint inhibitors to chemotherapy for SCLC is low, and only a few SCLC patients have shown a response to immune checkpoint inhibitors. Circulating tumor cells (CTCs) are tumor cells shed from solid tumor masses into the peripheral circulation and are key to tumor metastasis. Single-cell sequencing has revealed that the genetic profiles of individual CTCs are highly heterogeneous and contribute to the poor outcome and prognosis of SCLC patients. Theoretically, phenotypic analysis of CTCs may be able to predict the diagnostic significance of new potential targets for metastatic tumors. In this paper, we will discuss in depth the heterogeneity of CTCs in SCLC and the value of CTCs for the diagnosis and prognosis of SCLC and as relevant tumor markers in metastatic SCLC.

KEYWORDS
circulating tumor cell, heterogeneity, immunotherapy, small cell lung cancer, tumor micro-environment

Abbreviations: AC, atypical carcinoid; ADC, adenocarcinoma; ASCL1, achaete-scute complex homolog-like 1; CAN, copy number alterations; cfDNA, cell-free DNA; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; CTM, circulating tumor microemboli; CXCR4, chemokine C-X-C motif receptor 4; ECOG, Eastern Cooperative Oncology Group; ED-SCLC, extensive stage disease; EpCAM, epithelial cell adhesion molecule; FDA, Food and Drug Administration; G1, low-grade; G2, intermediate-grade; G3, high-grade; ITH, intra-tumor heterogeneit; LCNEC, large cell neuroendocrine carcinoma; LD-SCLC, limited-stage disease; LSQCC, lung squamous cell carcinoma; LTF, lineage-defining transcription factors; MMP-9, matrix metalloproteinase-9; MΦ*, macrophages; NCAM, Neural cell adhesion molecule; NECs, neuroendocrine carcinomas; NEN, neuroendocrine neoplasm; NETs, neuroendocrine tumors; NEUROD1, neurogenic differentiation factor 1; ORR, objective response rate; OS, overall survival; PDX, patient-derived xenografts; PFS, progression-free survival; POU2F3, POU class 2 structural domain transcription factor 3; PS, performance status; SCLC, small cell lung cancer; SLFN11, Schlafen family member 11; TC, typical carcinoid; VEGF, vessel endothelial growth factor; YAP1, yes-associated protein 1; μNMR, micro-nuclear magnetic resonance.

1 | INTRODUCTION

Lung cancer is one of the leading causes of death from cancer worldwide. Small cell lung cancer (SCLC), which accounts for 15%–20% of all lung cancers, is extremely aggressive with a very low cure rate [1]. SCLC is frequently found to have metastasized to sites outside the chest at the time of diagnosis, and most patients are in the extensive stage at the time of initial diagnosis. Most patients with extensive-stage disease eventually relapse, and few patients survive more than 5 years after diagnosis [2]. Treatment of SCLC has remained essentially unchanged over the past decades, and the survival rates for both localized and advanced diseases have not improved [3]. Chemotherapy remains the cornerstone of SCLC treatment, but resistance inevitably occurs [4].

Liquid biopsy is a new method for determining cancer cells or tumor DNA through the analysis of circulating tumor cells (CTCs), circulating tumor DNA, and exosomes [5, 6]. CTCs are tumor cells that are shed from the solid tumor mass into the peripheral blood circulation and spread to distant organs. CTCs are key to hematogenous metastasis. Single-cell sequencing revealed large numbers of variants in the genetic profile of individual CTCs; these variants are responsible for the poor outcomes and poor prognosis of SCLC patients. This article will provide insight into the heterogeneity of CTCs and the role of relevant tumor markers in metastatic SCLC.

2 | ENRICHMENT AND DETECTION OF CTCs

2.1 | Isolation and enrichment of CTCs

CTCs are present at extremely low levels in circulating peripheral blood; some studies have shown that there is only 1 CTC per 10^5–10^7 blood cells in cancer patients [7]. This poses a great challenge to isolate and enrich CTCs [8]. The expression of epithelial cell adhesion molecule [9] or the leukocyte marker CD45 [10] can be used to distinguish CTCs from blood cells such as leukocytes and provides a theoretical basis for the isolation of CTCs. Therefore, developing a method for the isolation and enrichment of CTCs with high capture efficiency, purity, and yield is a research goal. The current methods for the separation and enrichment of CTCs can be broadly classified into three categories: the first category includes methods exploiting the physical properties of CTCs, such as membrane filtration [11], density gradient centrifugation [12], and bidirectional electrophoretic field separation [13]; the second category includes methods using the biological properties of CTCs, such as immunomagnetic bead separation [14] and microfluidic chip separation method [15]; the third category includes enrichment methods based on the combination of physical and biological properties of CTCs. While various detection platforms with advantages have been developed using the properties of CTCs, the above-described separation methods using the physical and biological properties of CTCs and the combination of both are still the main methods.

2.2 | Detection technologies for CTCs

Various platforms are used to detect CTCs, and the Cell Search system is the only commercially available system approved by the U.S. Food and Drug Administration for the detection of CTCs [16]. Other innovative platforms such as the CTC Chip [17], microfluidic CTC capture chip [18], new micro-nuclear magnetic resonance [19], MagSweeper [20], and NanoVelcro CTC chips [21] have been reported in recent years. While these high-sensitivity CTC capture platforms have enabled fine molecular characterization of CTCs, the three-dimensional cell capture chamber has limitations for both high-throughput imaging and single-cell molecular analysis [22]. Other reported single-cell multigene approaches in breast cancer [23] and metastatic prostate cancer [24] are performed by manual selection, lysis, and reverse transcription to complementary DNA before parallel multigene expression profiling, thus greatly hindering their large-scale application [23, 25]. In contrast, the Cell Search system is useful for predicting the role of targeted therapies, improving prognosis, assisting tumor staging and grading of patients, monitoring treatment efficacy, and predicting tumor metastatic recurrence potential. This system is currently clinically approved for efficacy monitoring and prognostic assessment of patients with metastatic breast, prostate, and colorectal cancers, but not lung cancer. The survival of patients with metastatic breast cancer [26], prostate cancer [27], and colorectal cancer [28] is reduced when the serum CTC count is greater than 5, 5, and 3, respectively. Using this platform, CTC counting is used to assess the number of CTCs not only at baseline examination but also throughout the treatment course and/or after the completion of various treatment regimens [29]. The detection platform assesses various biological properties of CTCs, numerous relevant reports have indicated the utility of the Cell Search system in predicting response to tumor-targeted therapy, prognostic monitoring, and efficacy assessment. All in all, this system has the potential for comprehensive prediction of the SCLC patient outcome.

3 | HETEROGENEITY OF PERIPHERAL BLOOD CTCs

Tumor heterogeneity exists from molecular to organism level and is focused on the genome, transcriptome, proteome, and metabolome of lesions and tumors [30]. CTC heterogeneity exhibits high inter-patient/intra-patient heterogeneity in normal cancer cells such as pancreatic, breast, and colorectal cancers. CTC heterogeneity can be divided into morphological heterogeneity and phenotypic heterogeneity [31]. Genomic assessment of the heterogeneity between tumors and CTCs can be complementary. A combination of mutational testing of CTCs and tumor subjects would guide treatment more accurately compared with traditional clinical treatment [9]. With the role of targeted therapies in tumor treatment, screening tumor patients for abnormal genomic expression can help identify patients who may be resistant to treatment and provide more effective treatment strategies. Frequently multiple tissue biopsies for tumors are not feasible, but CTC-based liquid biopsies [32] can solve this problem, and sequencing and analyzing the entire genome carried by CTCs through single cells is of great utility in precision medicine. Stewart et al. [33] sequenced CTCs-derived xenografts from SCLC patients with chemosensitive or resistance and patients CTCs by single-cell RNAseq and found increased intratumor heterogeneity (ITH) including both heterogeneous expression of therapeutic targets and potential resistance pathways, such as epithelial-mesenchymal transition [9]. Similarly, sequence analysis of patient CTCs extracted directly from blood confirmed an increase in ITH after relapse. Morphologically, CTCs are also highly heterogeneous. A high variability in CTC value-added indices between patients was found by Ki67 staining [34], and heterogeneity in signaling pathways between CTCs of individual patients was also seen in single-cell analysis [35]. Single-cell sequencing-based transcriptome analysis revealed heterogeneity in the CTC subpopulation. Su et al. [36] confirmed that single-cell sequencing CTCs provide a noninvasive method for genome profiling and examining the evolutionary history of tumorigenesis, tumor evolution during treatment, and drug resistance potential, offering clinical stratification with SCLC. In a study by Magbanua et al. [37] to assess tumor heterogeneity at the level of CTCs, the expression profiles of individual CTCs were analyzed by multiplex aQPCR using a microfluidic-based dynamic array (Fluidigm), which showed higher levels of heterogeneity between single cells than mixed cells. Multidimensional scale analysis and clustering analysis revealed unique gene expression characteristics of CTCs in each patient.

4 | SCLC AND PULMONARY NEUROENDOCRINE TUMORS (NET)

The 2021 World Health Organization (WHO) classification categorizes neuroendocrine neoplasms (NENs) of the lung as NETs and neuroendocrine carcinomas (NECs). NENs include typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma of the lung (LCNEC), and SCLC [38]. NETs include low-grade (G1) TC and intermediate-grade (G2) AC. NECs include high-grade (G3) SCLC and LCNEC (Figure 1). Counting of mitoses is essential because it is important for histologic criteria for separating typical from ACs and carcinoids from the G3 SCLC and LCNEC [39]. TC, AC, and SCLC are currently treated with chemotherapy plus

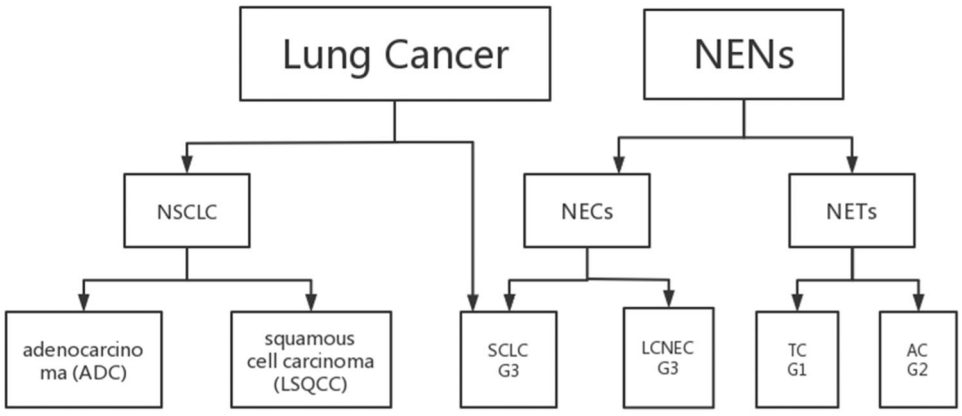

FIGURE 1 The classification of lung tumors. AC, atypical carcinoid; NEN, neuroendocrine neoplasm; NET, neuroendocrine tumor; SCLC, small cell lung cancer; TC, typical carcinoid.

immunosuppressive agents. The biological behavior of LCNEC is similar to that of SCLC. LCNEC is prone to recurrence and metastasis after surgery and resistant to chemotherapy, which ultimately leads to poor prognosis. The fourth edition of the WHO reclassified LCNEC from "large cell carcinoma" to "neuroendocrine tumor" [39]. In 2021, LCNEC was still classified as a neuroendocrine tumor [38]. SCLC is widely recognized as a profoundly malignant tumor derived from neuroendocrine cells. At present, the primary challenges in the treatment of SCLC primarily revolve around the absence of distinctive indicators for early detection of tumor recurrence and metastasis, the elevated rate of missed imaging in early-stage SCLC patients, the intricacy associated with tissue biopsy, the limited specificity and sensitivity of conventional blood tumor markers in early-stage SCLC patients and the fact that a majority of diagnosed SCLC patients have progressed to advanced metastasis stages, resulting in a substantial decline in their 5-year survival rates. Detection of the number of CTCs in liquid biopsy is simple and can dynamically monitor the gene expression of cancer cells. The use of CTCs in the detection of SCLC is beneficial for the early diagnosis and staging of patients and prognosis and is closely related to clinical staging.

5 | BIOMARKER STATUS OF CTCs AND SCLC

Schlafen family member 11 was recently identified as essential for the response of SCLC cell lines and patient-derived xenografts to chemotherapy [40]. Neural cell adhesion molecule (NCAM), also known as CD56, is a member of the immunoglobulin superfamily; the presence of NCAM on SCLC is consistent with the neuronal features of these tumor cells and has been studied as a marker for SCLC and a wide group of neuroendocrine cancers to which SCLC belongs [41]. However, there are few specimens available in biopsies, resulting in few tumor specimens for research to accurately determine the prognostic value of SCLC. The search for new markers targeting CTCs is a promising field and critical for developing individualized treatment. Elevated expression of chemokine C-X-C motif receptor 4 (CXCR4) is associated with shorter overall survival (OS) in NSCLC patients and a class of markers predicting poor prognosis in this disease [42]. The value of CXCR4 expression as a prognostic marker in SCLC is unclear. Salgia et al. [43] concluded that CTC count and CXCR4 expression in CTCs at baseline and after treatment are promising prognostic biomarkers for progressive SCLC. Su et al. [36] detected somatic mutations and copy number alterations (CNA) in SCLC by single-cell sequencing of CTCs during chemotherapy. CNA analysis on the basis of CTCs is attractive because it is not contaminated by normal cells. The presence of CNAs in recurrent chromosomes with significant differences between patients is a potential predictor and prognostic indicator, providing insight into biomarkers and developing a convenient approach to clinical disease treatment. Carter et al. [44] analyzed 88 CTCs isolated from 13 patients and generated a CNA-based CTC classifier. Using the classifier, the researchers found 83.3% of the cases were correctly assigned as chemically refractory or chemically sensitive. Liquid biopsy of peripheral blood CTCs will contribute to biomarker, prognostic, and predictive analyses in future SCLC studies, which may overcome the shortcomings of sampling smaller samples in this patient population and allow further evaluation of the efficacy of chemotherapy or drug therapy.

6 | HETEROGENEITY OF SCLC

SCLC exhibits inter-tumor heterogeneity, and the expression of lineage-defining transcription factors (LTF, such as ASCL1, NEUROD1, and POU2F2) seems to play a major role in this heterogeneity. The differential expression of four key transcriptional regulatory factors, achaete-scute complex homolog-like 1 (ASCL1; also known as ASH1), neurogenic differentiation factor 1 (NEUROD1), Yes-associated protein 1 (YAP1), and POU class 2 structural domain transcription factor 3 (POU2F3), is used to classify SCLC into four subtypes: SCLC-A, SCLC-N, and SCLC-P/Y based on 3K27ac chromatin immunoprecipitation followed by sequencing. In these subtypes, A, N, P, and Y represent ASCL1, NEUROD1, POU2F3, and YAP1, respectively; POU2F3, NEUROD1, and ASCL1 motifs are highly enriched at P, N, or A elements, respectively [45–47]. The subtypes of SCLC have been a research hotspot in recent years. For SCLC, the identification of subtypes has not established the strategies for individualized diagnosis and treatment. These findings indicate the coexistence of transcriptionally heterogeneous tumor cell populations with vulnerabilities. The shift in resistance mechanisms from chemosensitivity to chemoresistance with concurrent multiple resistance mechanisms is due to the coexistence of cell subpopulations with heterogeneous gene expression [33]. The discovery of transcriptional regulatory factor expression defining four SCLC subtypes may lead to significant breakthroughs in the future, and the identification of unique therapeutic targets for these four subtypes could help stimulate research of therapeutics for SCLC and ultimately improve the long-term prognosis of patients.

7 | THE IMPORTANT ROLE OF CTCs IN SCLC METASTASIS

The presence of heterogeneity in SCLC has prompted the exploration of novel diagnostic methods to address the limitations associated with conventional tumor genotyping. The mutational heterogeneity observed in SCLC is typically manifested in the tumor cell genome, with CTCs being the primary contributors to potential metastatic genomic alterations (Table 1). In theory, the use of liquid biopsy for SCLC has the potential to supplant the sequential biopsy of molecular biomarkers and mutations in metastases. This is because liquid biopsy, involving the capture and characterization of CTCs, can be accomplished through a straightforward blood sampling procedure. Moreover, CTCs can be consistently collected at various time intervals throughout the progression of the disease. The sequential acquisition of CTCs at multiple time points during the disease course can serve as a means to determine biomarkers or genes and CTCs can function as an alternative specimen to tumor tissue [48] (Table 2). The most studied approaches for liquid biopsy are cell-free DNA, CTCs, and exosomes [5]. In a study by Fiorelli [49] that included 77 patients with lung disease, CTCs were detected in the blood of 60 (90%) of patients with malignant cancer, while CTCs were detected in the blood of only 1 (5%) of the 17 patients without cancer. A study by Hou et al. [50] detected CTCs in 77 (85%) of 97 SCLC patients and circulating tumor microemboli in 25 (26%) patients, consistent with the high metastatic characteristics of SCLC. Wang et al. [51] showed a significant increase in CTC count with TNM stage (from stage I to IV), tumor size and aggressiveness, lymphatic metastasis, and histology in univariate analysis, indicating a positive correlation between CTC counts and tumor progression. Recent studies have shown the presence of CTCs in peripheral blood before tumor metastasis. Tay et al. [52] reported that 45 (60%) of 75 patients with limited-stage SCLC had detected CTCs, and CTC counts ≥15 were strongly associated with Eastern Cooperative Oncology Group Performance Status 1 and 0. The isolation and detection of CTCs in the bloodstream during cancer metastasis may offer the possibility to reveal potential mechanisms during cancer progression and therapeutic strategies for cancer (Figure 2).

8 | STUDY OF CTCs FOR ASSESSING THE PROGNOSIS OF LUNG CANCER PATIENTS

The number of CTCs is closely related to the prognosis of patients with various solid tumors, and CTC detection can be used as an adjunct to other clinical reference indicators to determine the prognosis of lung cancer (Table 2). Hou et al. [50] found that the OS rate of patients with ≥50 CTCs/7.5 mL of blood before chemotherapy was 5.4 months, while the OS in patients with <50 CTCs/7.5 mL the OS was 11.5 months. CTCs can be used as an independent prognostic factor in SCLC and a decrease in the number of CTCs less than 50 after the first round of chemotherapy predicted a poorer prognosis. Naito et al. [54] conducted a baseline examination and found that ≥2 CTCs were detected in 35 patients at baseline examination and ≥8 CTCs were found in the peripheral blood of patients at posttreatment or at relapse; the survival rate of patients with ≥8 CTCs was

TABLE 1 The role of CTCs in SCLC metastasis.

Study	Diease stage (LD-SCLC/ED-SCLC)	Platform	Main results
Hou et al. [50]	97 (31/66)	CellSearch System	85% CTCs and 26% CTM as well as the abundance of CTCs showed highly malignant of SCLC.
Wang et al. [51]	96 (64/32)	CellSearch System	The percentage of lung cancer patients detected CTCs increased from 5.9% (stage I) to 36.5% (stage IV). Tumor size and aggressiveness as well as local tumor size and aggressiveness as well as local lymphatic metastases were all associated with CTCs count at thresholds ≥1 or 5/7.5 mL.
Tay et al. [52]	75 LS-SCLC	CellSearch System	CTCs were detected in 60% LS-SCLC patients, this may reflect the high proportion of stage III (81%) versus stage I–II (15%) patients in detecting CTCs.
Aggarwal et al. [53]	50 (20/30)	CellSearch System	CTCs were present in 94% patients at baseline. The median CTCs number in ED patients was 91, whereas LD patients was only 1.

Abbreviations: CTC, circulating tumor cell; CTM, circulating tumor microemboli; ED-SCLC, extensive stage disease; LD-SCLC, limited stage disease; SCLC, small cell lung cancer.

TABLE 2 Summary of CTCs for assessing prognosis in SCLC.

Study	Diease stage (LD-SCLC/ED-SCLC)	Treatment	Platform	Main results
Hou et al. [50]	97 (31/66)	Chemotherapy	CellSearch System	≥50 CTCs/7.5 mL of SCLC blood detected worse PFS and OS. CTCs number and change in CTCs number after one cycle of chemotherapy to be an independent prognostic factor.
Tay et al. [52]	75 LD-SCLC	Chemotherapy	CellSearch System	≥15 CTCs at baseline independently predicted ≤1 year survival in 70% and ≤2-year survival in 100% of patients and with a median PFS and OS of 5.5 and 5.9 months, respectively.
Naito et al. [54]	51 (27/24)	Chemotherapy or chemoradioth-erapy	CellSearch System	The CTCs levels remained <8 patients' baseline and posttreatment or at relapse tended to show worse OS than those ≥8 cells.
Ju et al. [55]	99 (31/68)	Chemotherapy	ClearCell FX System	The ORR was 74.7% after received the first-line chemotherapy. The change of CTC counts was correlated with therapy response.
Shen et al. [56]	80 (40/40)	Chemotherapy	LT-PCR	83.8% of patients had positive CTCs count at baseline (CTCs ≥8.7 FU/3 mL). The reduction of CTC after two cycles of chemotherapy predicted chemotherapeutic response in SCLC.
Normanno et al. [57]	60 ED-SCLC	Chemotherapy	CellSearch System	CTCs were identified in 90% of patients at baseline. 89% following chemotherapy highly improved prognostic accuracy and was associated with a lower risk of death when CTC count reduced.

Abbreviations: CTC, circulating tumor cell; ED-SCLC, extensive stage disease; LD-SCLC, limited stage disease; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; SCLC, small cell lung cancer.

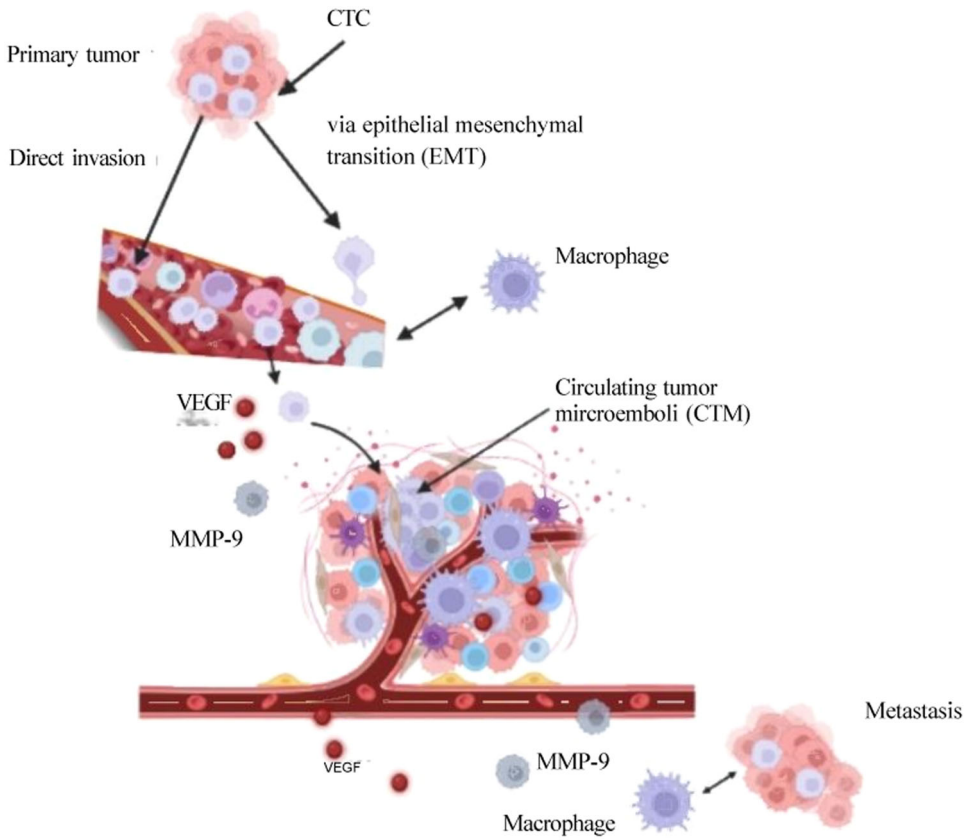

FIGURE 2 Representation of CTC-mediated metastasis in small cell lung cancer (SCLC). CTCs that have sloughed off the primary tumor and tumor core and circulated in blood vessels either directly or via EMT. Activation of macrophages (MΦ*) enhances extracellular matrix lysis and neovascularization by matrix metalloproteinase-9 (MMP-9) and blood vessel endothelial growth factor (VEGF), respectively. CTCs can be single intact CTCs, apoptotic CTCs, or small clusters of cells. Extravasion of CTCs is supported by the local recruitment of macrophages, which may then release a second wave after the formation of new blood vessels. The invading cells may then generate metastases. CTC, circulating tumor cell.

lower than that of patients with <8 CTCs. This result suggests that CTCs are highly detectable in SCLC, and higher levels of CTCs are strongly associated with poorer survival. Ju et al. [55] screened 14 SCLC patients who underwent first-line chemotherapy and the researchers performed CTC counts 1 week before and after two cycles of chemotherapy; the authors classified the patients as responsive, stable, and progressive using RECIST criteria 1.1. The results showed that the median CTC counts decreased by 6.96 in responsive patients and by 3.34 in stable patients, while in progressive patients, the median CTC count increased by 13.05, and treatment response was significantly associated with CTC count. Shen et al. [56] examined 80 SCLC patients, of whom 67 (83.8%) had positive CTC counts (CTCs ≥8.7 FU/3 mL). The median progression-free survival (PFS) was similar in patients with positive CTC counts and negative CTC counts (7.8 vs. 7.5 months); PFS and OS were significantly longer in patients with relatively low CTC levels than in patients with high CTC levels in the CTC count positive group (9.1 vs. 6.9 months), and high CTC levels showed prognostic significance only in patients with positive CTC counts. Thus, the number of CTCs and the altered concentration showed potential clinical efficacy in predicting PFS and OS in SCLC patients.

9 | CONCLUSION

The pursuit of novel markers specific to CTCs is a critical research focus. Through improvements of CTC sorting, enrichment, and detection methodologies, exploring alterations in CTCs at the molecular level may improve our comprehension of the biological attributes underlying tumor progression and metastasis. Multiple investigations have demonstrated the clinical importance of CTCs for the early diagnosis of SCLC and the surveillance of SCLC recurrence and prognosis. Exploring the molecular phenotypic differences of CTCs in different individuals and the heterogeneity of CTCs in the same

individual will also be a focus of future research. In the future, the clinical application of CTC molecular characterization may help identify CTC subpopulations/genetic features, thus enabling patient classification, determining SCLC metastasis and prognosis, predicting the role of targeted therapies in SCLC patients, and even helping to assess patient clinical trial eligibility. The molecular characterization of CTCs has helped to bring SCLC to the forefront of precision medicine. Currently, the detection of CTCs still faces many problems that need to be solved, such as the fact that CTCs are only 0.01% of peripheral blood [58], but the number of CTCs in SCLC patients is high and therefore the study of CTC in SCLC has certain advantages. In addition, further research is needed to determine whether the heterogeneity of CTCs will cause inaccurate diagnoses. With continuous research, the application of CTCs in the diagnosis of SCLC has strong potential and will be explored to improve the diagnosis, metastasis, treatment, and prognosis of SCLC patients in the future.

AUTHOR CONTRIBUTIONS

Qunxia Wang: Conceptualization (lead); data curation (lead); formal analysis (lead); writing—original draft (lead); writing—review and editing (lead). **Li-Ming Tan**: Project administration (lead); resources (lead); supervision (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

ETHICS STATEMENT

Not applicable.

INFORMED CONSENT

Not applicable.

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