

## Circulating Tumor Cell and Cell-free Circulating Tumor DNA in Lung Cancer

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Circulating tumor cells (CTCs) are tumor cells that are separated from the primary site or metastatic lesion and disseminate in blood circulation. CTCs are considered to be part of the long process of cancer metastasis. As a 'liquid biopsy', CTC molecular examination and investigation of single cancer cells create an important opportunity for providing an understanding of cancer biology and the process of metastasis. In the last decade, we have seen dramatic development in defining the role of CTCs in lung cancer in terms of diagnosis, genomic alteration determination, treatment response and, finally, prognosis prediction. The aims of this review are to understand the basic biology and to review methods of detection of CTCs that apply to the various types of solid tumor. Furthermore, we explored clinical applications, including treatment monitoring to anticipate therapy resistance as well as biomarker analysis, in the context of lung cancer. We also explored the potential use of cell-free circulating tumor DNA (ctDNA) in the genomic alteration analysis of lung cancer.

**Key Words:** Neoplastic Cells, Circulating; DNA, Neoplasm; Lung Neoplasms; Biology; Methods

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## INTRODUCTION

Lung cancer remains the main cause of mortality among patients with cancer worldwide.<sup>1,2</sup> Among American patients with cancer, it is estimated that lung cancer is responsible for the mortality of 28% male and 26% female patients.<sup>3</sup> Efforts have been made to detect lung cancer as early as possible by methods such as chest computerized tomography (CT) scanning,<sup>4-6</sup> sputum cytology analysis,<sup>7,8</sup> biomarker analysis,<sup>9,10</sup> circulating tumor cell (CTC) detection,<sup>11</sup> and circulating cell-free tumor DNA (ctDNA) detection.<sup>12,13</sup> An average solid tumor may release an estimated million cells per day into the bloodstream and although most dispersed cancer cells do not survive, the cells that do survive clearly pose an existential threat to the host organism.<sup>14</sup>

Detection, monitoring, and molecular investigation of CTCs and ctDNA offer a meaningful and noninvasive way for the detection of the early phase of the disease, prognosis

prediction and assessing therapeutic response in patients with lung cancer.<sup>15,16</sup> As a so-called 'liquid biopsy', single tumor cell analysis and ctDNA analysis hold significant potential in providing understanding into lung cancer biology and the cancer spreading process.<sup>17</sup> Until now CTC characterization and enumeration has been extensively studied in several cancers such as colorectal carcinoma,<sup>18,19</sup> prostate cancer,<sup>20,21</sup> breast cancer,<sup>22,23</sup> ovarian cancer,<sup>24,25</sup> head and neck carcinoma,<sup>26,27</sup> brain cancer,<sup>28-30</sup> gastric cancer,<sup>31,32</sup> hepatocellular carcinoma,<sup>33-35</sup> renal cell carcinoma,<sup>36</sup> mesothelioma,<sup>37,38</sup> and lung cancer.<sup>39,40</sup> In general, the objectives of studies on CTCs in solid tumor include (a) risk prediction for metastatic progression and relapse, (b) staging determination and real-time observation of response to therapies, (c) determination of therapeutic targets and mechanisms of resistance, and (d) understanding metastasis process in solid tumor patients.<sup>41</sup>

Circulating ctDNA can be identified in lung cancer patients with the polymerase chain reaction (PCR) assay and

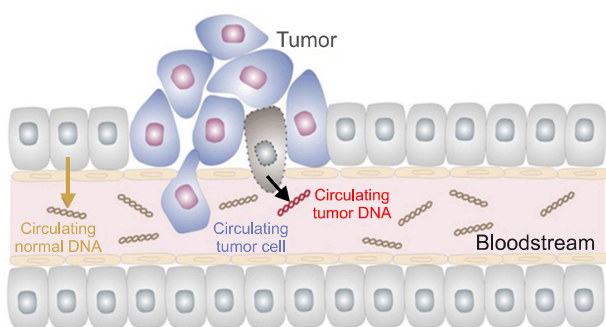
high plasma DNA levels could also identify high-risk persons for lung cancer screening.<sup>42</sup> Moreover, ctDNA in patients with lung cancer displays genetic and epigenetic differences common for tumors including oncogene activation, chromosome loss, and tumor-suppressor gene silencing by methylation.<sup>43</sup> Studies have shown that ctDNA levels were associated with tumor grade, tumor stage, lymph node spreading, the number of metastatic sites, tumor response to treatment and, finally, survival in patients with non-small cell lung cancer (NSCLC).<sup>44</sup>

## METHODS

The purpose of this review is to discuss the basic concept and methods of detection of CTCs and ctDNA in solid tumors generally. We also aimed to see how these advancements of knowledge have been applied in assessing patients with lung cancer, both small NSCLC as well as small cell lung cancer, based on accumulated evidence. A review of the published peer-reviewed reports in the Medline database up to October 2015 was performed. The terms used to gather relevant original articles, reports, and reviews were: circulating tumor cells, circulating cell-free tumor DNA, lung cancer, and epithelial-mesenchymal transition. A narrative review format was used to present the information collected.

## DEFINITIONS

CTCs, with a spectrum from one cell to aggregates consisting of 2-5 cells, are tumor cells that detach from either a primary lesion or a metastatic site and spread in the peripheral blood as the cellular seed of metastasis (Fig. 1).<sup>45,46</sup> 1 gram of tumor tissue, about 1 million tumor cells, can disseminate daily into the blood stream.<sup>47</sup> CTCs in blood circulation derive from solid tumors. They are involved in the metastatic spread to distant organs that leads to the formation of secondary sites of the disease.<sup>48</sup> Meanwhile, circulating tumor microemboli (CTM) is a tumor cell aggregate that circulates in the blood.<sup>49</sup> Another term is disseminating tumor cells (DTCs), which are defined as a settlement of CTCs in secondary organs and may stay in a quiescence state or may cause an observable metastasis.<sup>50</sup> CTCs and DTCs in blood and bone marrow are considered as potential metastases-inducing cells.<sup>51</sup> At least one CTC per 3 ml of blood was reported in 17 (65%) and one CTM per 3 ml of blood in 15 (58%) of 26 NSCLC cases.<sup>52</sup>



**FIG. 1.** Circulating tumor cells (CTCs) and cell-free circulating tumor (ctDNA) originated from the primary tumor or metastatic lesion circulates in the blood. Reprinted with permission from Sysmex Inostics Corp.

inating tumor cells (DTCs), which are defined as a settlement of CTCs in secondary organs and may stay in a quiescence state or may cause an observable metastasis.<sup>50</sup> CTCs and DTCs in blood and bone marrow are considered as potential metastases-inducing cells.<sup>51</sup> At least one CTC per 3 ml of blood was reported in 17 (65%) and one CTM per 3 ml of blood in 15 (58%) of 26 NSCLC cases.<sup>52</sup>

Cell-free circulating tumor DNA (ctDNA) may come from primary or metastatic tumor deposits and is enriched for detection of metastatic precursors.<sup>53</sup> ctDNA genotyping has some advantages over CTCs because (1) CTCs must be separated from the much more abundant hematologic cells in the blood requiring comprehensive laboratory facilities to obtain a viable population of CTCs for study; (2) CTCs in circulation encounter substantial apoptosis and fragility leading to variability between different CTC assays; (3) most of the ctDNA genotyping methods require a minimum of special handling and do not depend on special equipment, and lastly; (4) ctDNA could be processed at the same time with plasma DNA from normal cells, which always exists in the bloodstream.<sup>54</sup>

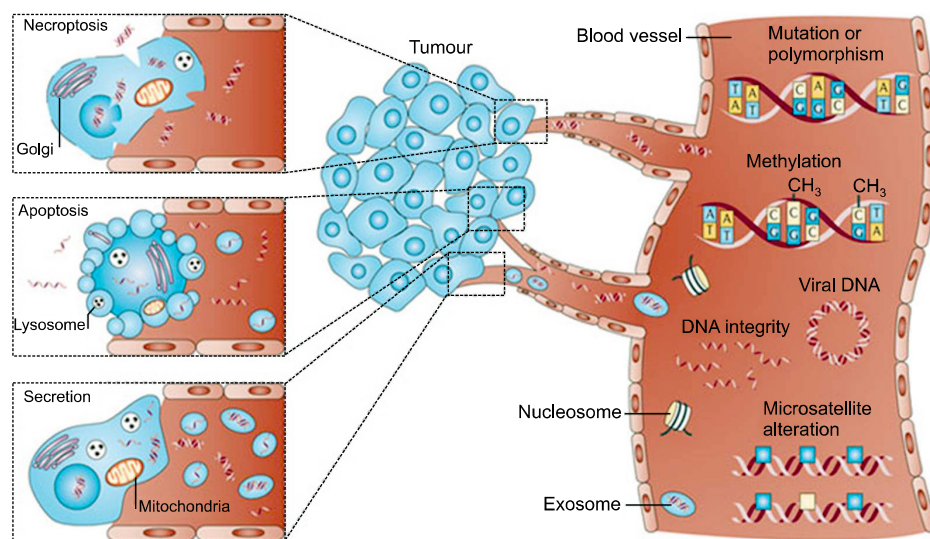
## FORMATION OF CTC AND CTDNA

CTC takes part in the long process of tumor metastasis, it is believed that metastasis is started by a sub-group of CTC seen in the blood of patients.<sup>55</sup> Metastasis consists of the two steps: The first steps is the displacement of a cancer cell to a distant site, while the second step is related to the capacity of the cancer cell to generate a metastatic foci at that distant organ.<sup>56</sup> To detach from the primary tumor, cancer cells needs to undergo a cellular process known as the epithelial-mesenchymal transition (EMT).<sup>57</sup> EMT allows the tumor cells to gain motility and migratory capacity which results in penetration into bloodstream and circulation as CTC.<sup>58</sup>

The release of DNA into circulation is a common phenomenon in cancer patients due to apoptotic and necrotic processes characteristic of tumor cells.<sup>59</sup> Methylation, mutations, microsatellite alterations, DNA integrity, and viral DNA can be studied in the ctDNA, as well as contributing factors of tumor DNA released into circulation which are tumor burden and tumor cell proliferation (Fig. 2).<sup>60</sup> Cell-free DNA found in lung cancer patients originates primarily from tumor development and necrotic malignant cells that have been enveloped by macrophages rather than the chronic inflammatory response.<sup>61,62</sup>

## METHODS OF DETECTION

CTC identification, enumeration, and molecular analysis are very difficult because CTCs occur typically with a frequency 1 per  $10^{6-7}$  leukocytes and the amount of available sample is very limited.<sup>63,64</sup> Many methods have been tested to detect and characterize CTCs in lung cancer patients.<sup>65</sup> Most of the current strategies to enumerate CTC are generally based on CTC markers and size. In the



**FIG. 2.** Circulating cell-free tumor DNA exist in the blood in various forms, and could provide information such as mutations, DNA integrity, methylation, viral DNA, and microsatellite alterations. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer 11: 426-437, copyright (2011).

surface marker-based strategy, CTC detection is based on epithelial cell adhesion molecule (EpCAM) and keratin markers.<sup>66</sup> One of the methods that are commonly used to enumerate CTC is CellSearch<sup>®</sup> CTC Test which has been shown to consistently capture clinically important CTCs.<sup>67</sup> So far, only the CellSearch<sup>®</sup> system has been approved by the FDA for CTCs detection of epithelial origin in whole blood and to assess prognosis.<sup>68</sup> CellSearch<sup>®</sup> isolates CTCs by using the EpCAM-based enrichment technique.<sup>69</sup> The nanotechnology approach in CTCs detection has also been studied. Gazouli and colleagues demonstrated a sensitive assay that combines magnetic beads, coupled with EpCAM and CK19 antibodies, and quantum dots (QDs) fluorescence detection for the detection of as little as 10 CTCs per ml blood samples.<sup>70</sup> However, because CTCs of lung cancer often present non-epithelial characteristics and lose their epithelial markers during the EMT process, this detection method can become ineffective and result in poor sensitivity.<sup>51,71</sup> In breast cancer, loss of EpCAM is accompanied by the upregulation of caveolin1 (CAV1) in CTC. CTC detection devices using CAV1-EpCAM conjugated beads give significantly improved throughput compared to EpCAM only.<sup>72</sup> However, there has been no reporting about the use of CAV1-EpCAM-based CTC detection in lung cancer.

To address the limitation of EpCAM-based strategies, efforts to classify CTCs based on their EMT markers and to elucidate the heterogeneity of CTC populations have been made.<sup>73</sup> Recently, the CanPatrol CTC enrichment method was used to detect CTCs using EMT markers in various types of malignancies.<sup>74</sup> This technique might offer a solution because EpCAM-based enrichment methods have led to missed CTC detection that has lost their EpCAM expression due to EMT.<sup>75</sup>

As for the size-based CTC detection, most commonly, microfluidic chips are used, which involves filtration of micro-channels to isolate the larger CTCs from the other blood components.<sup>76</sup> One study reported that an ultra-high-throughput spiral microfluidic biochip device could enrich CTC

with a fast processing time (7.5 mL blood within 10 min), 100% detection rate (10/10) of blood samples collected from patients with advanced-stage metastatic lung and breast cancers, and was able to capture 20 to 135 CTCs/mL with high purity of 1 CTC out of every 30-100 white blood cells.<sup>77</sup> Another sized-based CTC detection, microcavity array (MCA) system connected with a small device for CTC detection independent of EpCAM expression, has been reported to be successful in isolating CTC populations from patients who had been determined as CTC negative by the CellSearch system.<sup>78</sup> The MCA system is a microfabricated nickel filter with a rectangular MCA (10(4) cavities/filter) and the shape and porosity of the MCA were customized to efficiently identify small tumor cells on the microcavities under low flow resistance while at the same time allowing other blood cells to pass through.<sup>79</sup>

Ligand-targeted PCR (LT-PCR) is a system where CTCs, that were enriched from erythrocytes and leukocytes, were marked with a conjugate of a tumor-specific ligand and a synthesized oligonucleotide followed by quantitative PCR analysis.<sup>80</sup> LT-PCR provide quantification of CTC in NSCLC patients with greater sensitivity, about 80% sensitivity of stage I/II, 67% sensitivity of stage III, and 93% sensitivity of stage IV NSCLC.<sup>81</sup> An assay that identified live cells using an adenoviral probe that detected elevated telomerase activity present in almost all cancer cells, but not in normal cells, could detect CTC in 65% of lung cancer patients.<sup>82</sup> ISET (RareCell Diagnostics, Paris, France) is a technology that does not rely on the tumor marker expression, in which CTCs are captured by filtration independent of the tumor-related markers, because of their large size compared to circulating white blood cells.<sup>83</sup> Filtration using the ISET system could detect larger numbers of CTCs, including epithelial marker negative cancer cells and isolate CTM and allow molecular analysis.<sup>84</sup> The challenge is not limited to detection only but also culturing. If we could culture and expand the number CTCs ex vivo, it would allow us to examine genotype and phenotype of the tumor cells. In this re-

gard, the Thermo-responsive NanoVelcro CTC purification system has been investigated for culture expansion of CTC from NSCLC.<sup>85</sup> The Thermo-responsive NanoVelcro is the third generation of NanoVelcro in which agent-coated nanostructured substrates immobilized CTC while maintaining cells viability and molecular integrity.<sup>45</sup>

Calculation of ctDNA could assist in objective response assessments, identification of subclinical residual disease and provide a noninvasive approach for tumor genotyping.<sup>86</sup> The quantity of plasma ctDNA can be determined using quantitative Real-Time PCR.<sup>87</sup> It has been shown to reach the level of 12.8 ng/mL in lung cancer.<sup>12</sup> Novel methods called the 'cancer personalized profiling by deep sequencing' (CAPP-Seq) for quantifying ctDNA can detect ctDNA in all the patients with stage II-IV and in half of the patients with stage I NSCLC.<sup>88</sup>

## CLINICAL APPLICATIONS

In the future, CTC applications will not be limited to enumeration. Daily characterization using a high-content approach that studies protein expression, morphometrics, genotype profiling and eventually prediction of the metastasis of the disease from the primary tumors to distant organs will become possible.<sup>89</sup> Circulating cancer cells may be reliable markers in the diagnostic procedures of lung lesions.<sup>90</sup> Even if without obvious tumor nodules, the CTCs can be captured in patients with COPD and non-detectable lung malignancy.<sup>91</sup> Generally, the rate of CTC detection is positively correlated with NSCLC patients' overall stage and metastatic status.<sup>92</sup>

It has been demonstrated that CTC investigation through serial blood sampling could help provide personalized medicine for SCLC.<sup>93</sup> Circulating tumor cells, prior to the initiation of chemotherapy, are valuable predictors of specific progression-free survival (PFS) in Stage III patients with SCLC.<sup>94</sup> CTC enumerations could also be used for overall survival (OS) and PFS prediction. It has been shown that patients with no CTCs detected before treatment have a prolonged PFS and OS and show a greater percentage of stable disease compared to patients with 1-3 and > 3 CTCs which impose a higher risk of having progressive disease.<sup>95</sup> In terms of evaluation after treatment, patients with positive detection of CTCs after therapy show a poorer response to therapy.<sup>96</sup>

## BIOMARKER ANALYSIS OF CTC

Real-time PCR and melting curve analysis have been used to find sensitizing EGFR mutations in blood cells enriched in CTC.<sup>97</sup> Analysis of EGFR can also be performed in captured CTCs by immunofluorescence staining.<sup>98</sup> EGFR mutation analysis on DNA recovered from CTCs showed high sensitivity, including identification of the T790M secondary mutation which confers drug resistance.<sup>99</sup> Using ctDNA specimens, it was found that follows up to the EGFR mutations in the circulation allow for identification of the

T790M mutation for up to 344 days before patients suffered from disease progression.<sup>100</sup> Consistent with this finding, studies that quantitatively detected activating EGFR mutations and the resistant T790 secondary mutation showed a 72.7% detection rate of EGFR mutation in all lung cancer patient enrolled in the study. The detection rate of the T790M mutation among patients who experienced progressive disease after EGFR-TKI treatment was 43.5%. The BEAMing (beads, emulsion, amplification, and magnetics) method was used for detection.<sup>101</sup>

As for KRAS mutation, it was reported that the colorimetric membrane array analysis technique could identify an activated KRAS mutation from CTC in various cancers.<sup>102,103</sup> This finding was then followed by the establishment of the platform-weighted chemiluminescent membrane array (WCHMA) which is able to detect the KRAS mutation from minimally 3 CTCs/ml blood with a sensitivity of 93% and a specificity of 94%.<sup>104</sup> In the WCHMA method, the best intensity value for each gene, for example, KRAS, is calculated by the linear value of the chemiluminescent emission and is more accurate than the color concentration readings in colorimetric membrane array method.<sup>105</sup> However, recent evidence suggests that ctDNA was superior and preferable as type of specimen for KRAS mutation analysis in lung malignancies compared to CTC DNA because it could lead to greater mutation detection than CTCs.<sup>40</sup>

CTCs have also been used clinically to diagnose echinoderm microtubule associated proteins like 4-anaplastic lymphoma kinase (EML4-ALK) gene rearrangement in NSCLC to select patients who are eligible to receive ALK inhibitor.<sup>106</sup> ALK rearrangement could be determined in CTCs of ALK-positive NSCLC patients by applying a filtration technique and filter adapted fluorescence *in situ* hybridization (FA-FISH).<sup>107</sup> FA-FISH comprises a filter for detecting CTCs that have been previously enriched by blood filtration, followed by preparation of a filter spot for cells fixation and the cells analysis using the FISH assay.<sup>108</sup> Moreover, ALK protein expression could also be observed in CTCs isolated from lung cancer patients by immunocytochemistry.<sup>109</sup> FISH analysis provided sufficient positivity of EML4-ALK gene rearrangement detection from 10-1535 CTCs/mL and only required 7.5 mL of patient blood samples.<sup>110</sup>

Taken together, these advancements show that genetic modifications in solid cancers can be characterized by massively parallel sequencing of ctDNA shed from cancer cells into plasma. Repeated biopsies to investigate genomic dynamic changes as a consequence of therapy are not easy, invasive and may be confounded by the heterogeneity nature within the tumor.<sup>111</sup> The potential of ctDNA as a replacement of metastasized tumor biopsy becomes more evident because, in some patients, mutation expression analysis from metastasis lesion biopsies failed because of insufficient biopsy sizes, but was successful in all plasma ctDNA samples.<sup>112</sup> However, despite the promising application and their applicability for employing CTCs to diag-

nose genomic changes and monitor responses to therapies (ie, as a liquid biopsy) in lung cancer, these technologies have been limited by significant hurdles, such as complex systems that requires high-level laboratory capacity, contaminated blood cells, and undefined gold-standard method, and have not compiled momentum to add tissue-based diagnostics.<sup>113</sup>

## CONCLUSIONS

CTCs are tumor cells that can be captured through a liquid biopsy from blood and can be genetically and phenotypically studied to provide important data for guiding cancer therapy. The clinical values of CTCs as a biomarker for early-phase cancer screening, diagnosis, the prediction of treatment response, prognosis, and stratification have been widely explored in recent years.<sup>114</sup> It is expected that an understanding of CTC biology and its implications in their clinical application will help clinicians in the treatment of lung cancer. Despite high sensitivity and specificity, technological issues have limited the broad clinical utility of the method. It may need several years for CTC detection to become applicable in the clinic for the routine diagnosis of cancer. Obviously, further investigation is required to establish standardized techniques for sample collection, processing, and analysis.

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## CONFLICT OF INTEREST STATEMENT

None declared.

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