[Frontiers in Bioscience 19, 896-903, June 1, 2014]

Methods for detection of circulating cells in non-small cell lung cancer

Yi Han[1,2], Chongyu Su[1,2] , Zhidong Liu[1,2]

[1]Department of Thoracic Surgery II, Beijing Chest Hospital, Capital Medical University, Beijing, China; [2]Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing, China

TABLE OF CONTENTS

1. ABSTRACT

Circulating tumor cells (CTCs) from peripheral blood have been detected in most epithelial malignancies. CTCs are very heterogeneous and can be captured via different technologies based on their physical and biological properties. The detection rates have varied depending on the technology used for enumeration. Detection, monitoring, and molecular analysis of CTCs provide a powerful and noninvasive approach for the detection of early disease, assessing prognosis and therapeutic response in cancer patients. Non-small cell lung cancer (NSCLC) is one of the most lethal malignancies in humans. Compared with other solid tumors, the number of CTCs in NSCLC is relatively low. Nevertheless, NSCLC is a particularly important disease for CTC evaluation for prognostic purposes because of the lack of a reliable protein-based tumor marker. Molecular analyses of CTCs have provided new insights into the biology of metastasis with important implications for the clinical management of cancer patients. We review current and emerging technologies for CTC detection, with a focus on enrichment and molecular analysis of CTCs, and their potential clinical applications in NSCLC.

2. INTRODUCTION

Lung cancer has become the leading cause of cancer-related deaths worldwide in recent years(1). Metastasis is the most vital factor causing the adverse prognosis of cancer patients, not least in lung cancer, by compromising the function of an organ. 90% of cancer patients do not die because of their primary tumor; they are killed by metastatic disease(2-4). Malignant cells localized within the primary tumour can invade through the basement membrane and migrate into circulation, finally travel to a distant tissue and establish a separate secondary tumour site in a new environment of a host organ, such as bone marrow, liver, lung or brain(5-7). Unfortunately, the current high-resolution imaging technologies and serum tumor markers are incapable of detecting the early spread of tumor cells, the ongoing metastasis as early as possible, which prevented potentially effective early intervention. Ultrasensitive immunocytochemical and molecular assays have been recently developed to detect disseminated tumor cells (DTCs) in the bone marrow and circulating tumour cells (CTCs) in the peripheral blood of cancer patients. Application of DTCs analyses has provided new insight into the biology of metastasis with important implications

for estimating prognosis and the clinical management of the patients with breast, colon, lung and prostate cancer(6, 8-11). Peripheral blood analyses, however, are more acceptable and convenient for patients with solid tumors than invasive bone marrow sampling which is the standard of care in patients with leukemia or lymphoma(10). Enumeration of CTCs present in the peripheral blood of patients with malignant cancer could prove a useful tool for detection, characterization, prognosis and treatment monitoring(12). CTCs occur at very low concentrations of one tumor cell in the background of millions of blood cells, in that only a few CTCs are contained per milliliter of peripheral blood(13, 14). This presents a tremendous challenge for the development of a sensitive and specific detection method for CTCs, as well as for the clinical application. However, there have been major technological advances in recent years. CTCs can now be reliably identified in the peripheral bloodstream of cancer patients with metastatic disease, and their biological significance is being revealed. At least 13 other methods have been described to identify them by differences in biological and physical properties of CTCs (14). The potential applications of CTC enumeration and characterization could facilitate several key areas of cancer therapeutics, such as guiding prognosis, measuring response to anticancer therapy, selecting patients for adjuvant chemotherapy, and detecting recurrent disease(14-16). Only a few reports have been published on CTCs in non-small cell lung cancer (NSCLC) (17-22). When comparing data with the same technology, incidence of CTCs in NSCLC was lower compared with other tumors as reported by the CellSearch method(23). NSCLC is a particularly important disease for CTC evaluation for prognostic purposes because of the lack of a reliable protein based tumor marker(24). This review will discuss the most commonly methodology for detection of CTCs and their clinical utility with particular reference to NSCLC.

3. NON-SMALL CELL LUNG CANCER (NSCLC)

Lung cancer is a major health challenge worldwide, ranking at one of the most common malignancies in the world, and its incidence is rising in many countries. Lung Cancer is the most frequent cancer in terms of incidence (1.35 million new cases or 12.4% of the world total) and the deadliest cancer with more than 1.18 million deaths or 17.6% of the total (1, 25). NSCLC accounts for the 80% of all lung cancers with a gloomy prognosis, and less than 15% of patients surviving beyond 5 years(26). NSCLC is divided into 3 types according to histological characteristics: squamous cell carcinoma (SCC; 25-30%), adenocarcinoma (AC; 32-40%), and large cell carcinoma (LCC; 8-16%). All of these subtypes are highly lethal and hard to distinguish clinically(27). Despite advances in surgery, chemotherapy, and radiotherapy over the last decades, the death rate from lung cancer has remained largely unchanged, which is mainly due to lack of effective diagnosis biomarker and occurrence of metastases in distant organs for the vast majority of lung cancer patients. In many cases, distant metastases have already developed by the time of the diagnosis of the primary lung lesions, and therefore the majority of patients requires systemic therapy (26, 28). Histological differentiation and staging of lung cancer is mandatory at time of diagnosis for the individual therapeutic stratification(29). In many patients, biopsies for the histopathological subtyping cannot be taken due to multimorbidity and instable clinical conditions of the patient or unfavorable localization of the tumor (26, 29). Meanwhile, unlike CEA in colorectal carcinoma and PSA in prostate cancer, tumor markers are not widely accepted in making treatment decisions for patients with lung cancer. At presentation, decisions in lung cancer treatment are based mainly on imaging rather than on fluid based biomarkers. Therefore, nearly 50% of early-stage NSCLC patients will relapse or develop distant metastases within 5 years even after potentially curative treatment as surgical removal of tumor mass(30). Early detection has consistently been shown as the best defense against any cancer, whereas the stage at diagnosis and the available options for curative surgery remain the most important prognostic factors. In lung cancer, for improvement of the survival of the patients with NSCLC, it is extremely important to establish feasible diagnostic methods of not only as accurate predictors for possible formation of metastasis but real-time monitoring of patient progress over the course of therapy.

4. CIRCULATING TUMOR CELLS (CTCS)

CTCs have attracted much interest in cancer research as a potential biomarker, as a guild for treatment decision making in cancer patients and as a means of studying the process of metastasis (31-33). The “seed and soil” hypothesis, which predicted the existent of CTCs in the peripheral blood of cancer patients, was firstly documented by Paget in 1889(34), which were also theorized by other nineteenth century physicians, with some even identifying the existence of malignant cells in the circulation of cancer patients(35). CTCs are defined as tumor cells disseminating from either primary sites or metastases, circulating freely in the peripheral blood of patients, at least some of which are ultimately capable of forming distant metastases, whereas extremely rare in the healthy people(6, 7, 36). Highly aggressive CTCs may not only establish metastases in distant organs, but also be capable of self-seeding back to their original organs (37). The prognosis of carcinoma patients, even with small primary tumors, is mainly determined by the CTCs from the primary site to distant organs such as bone marrow, liver, lungs, or brain, and the subsequent outgrowth of these cells in their new microenvironment(35, 38). Despite of detection rates varied depending on the technology used for enumeration, it has now been shown that CTCs can be harvested from blood in various cancer subtypes, such as breast, colon, lung, and prostate cancers(39-42), which strongly suggested that CTC detection had huge potential of assisting malignancy diagnosis, estimating prognosis, monitoring disease recurrence, and response of the treatment(14, 36). Moreover, characterization of CTCs offers new perspectives for researchers to renew our understanding of the evolution of tumour genomes during treatment and disease progression, and shed new light on the underlying mechanism of the biology of treatment resistance and metastasis.

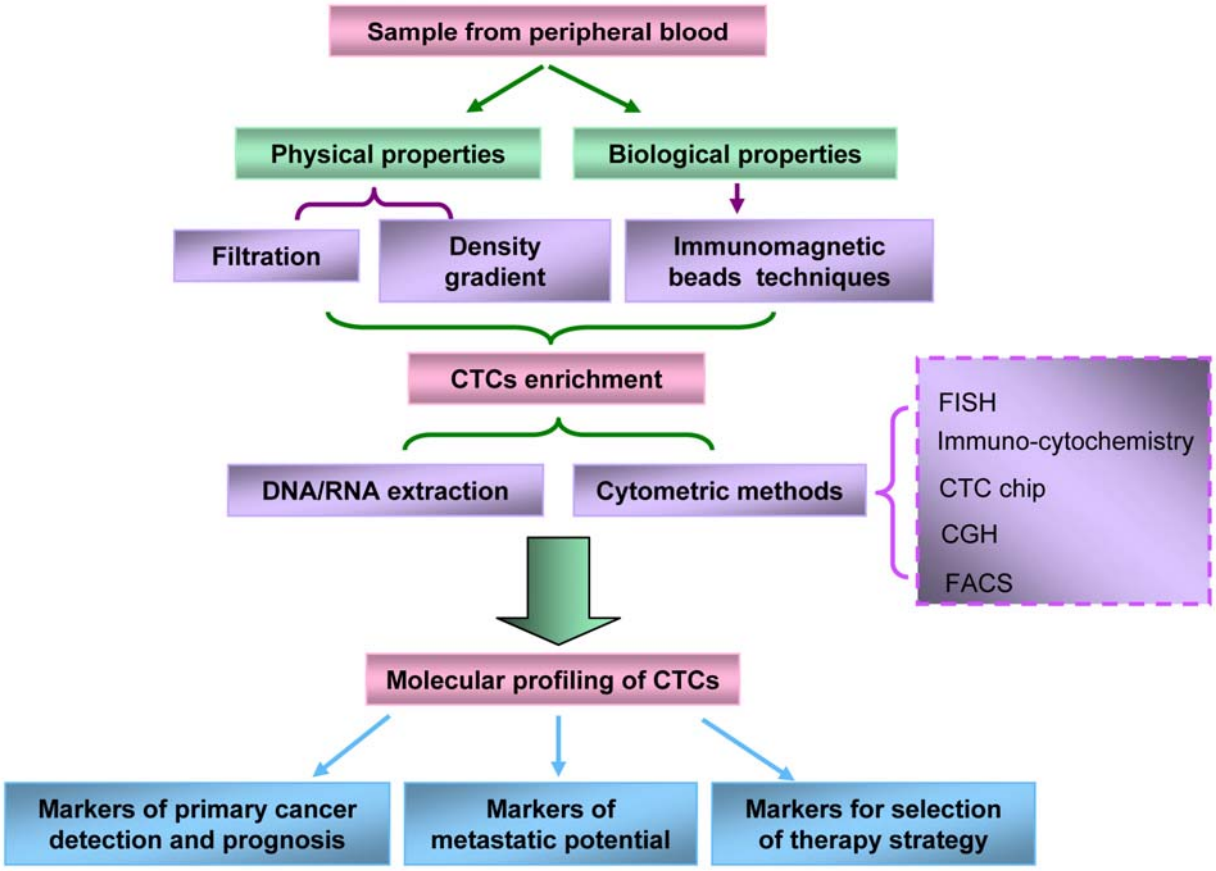

Figure 1. Representative methods for enrichment and analysis for circulating tumor cells. FISH fluorescence *in situ* hybridization, CGH comparative genomic hybridization, FACS fluorescence-activated flow cytometry.

The detection of CTCs in peripheral blood of cancer patients holds great promise, and much of the research over the past few decades has been focused on the development of reliable methods for CTCs enrichment and identification(12, 13, 16). It is known that even in patients with advanced cancers, CTCs exist in extreme rarity in patient blood, vastly outnumbered by normal blood cells (43-46). The identification of CTCs must be sensitive and specific enough to distinguish the malignant cells from all the other circulating non-tumor hematopoietic cells, which are usually combined with enrichment procedures. CTCs enrichment includes amounts of technologies based on the different properties of CTCs that distinguish them from the surrounding normal hematopoietic cells, including physical properties (size, density, electric charges, deformability) and biological properties (surface protein expression, viability, and invasion capacity)(13). Techniques for physical properties, which based on the hypnosis that CTCs are often larger than normal leukocytes, include filtration of tumor cells by size through special microporous polycarbonate filters, such as the ISET (Isolation by Size of Epithelial Tumor Cells)(47, 48), density gradient centrifugation (Ficoll, OncoQuick)(49-51) or a novel three-dimensional microfilter(52). Due to the lack of markers, these methods are generally considered not highly sensitive and poorly specific (53-55). Biological properties are mainly used in immunological procedures with antibodies against either tumor-associated antigens (positive selection) or the normal leukocyte antigen CD45 (negative selection) (Figure 1). Until now, the most successful approaches have made use of the fact that epithelial cell commonly express the cell adhesion protein Epithelial cell adhesion molecule (EpCAM), which is absent in normal blood cells. The majority of studies have employed the CellSearchTM System (Veridex LLC, Huntingdon Valley, PA, USA) which is an immunomagnetic detection method employing magnetic beads coated with anti-EpCAM antibodies(13). However, all EpCAM-based enrichment systems have the same limitation(56); EpCAM can be downregulated during epithelial-to-mesenchymal transition (EMT), which is a key developmental program that is often activated during cancer metastasis and invasion(57, 58). In addition, EpCAM is not necessarily expressed by all types of tumors(59, 60).

Other general limitation of CTCs detection is that there is no single marker that will reliably identify CTCs and be used independently for various types of tumors(14, 16, 35). This limitation applies not only to different genetic characteristics among different tumor tissues, but also to different genetic characteristics in the same tumor tissue (61-63). Meanwhile, tumor cells in circulation are

heterogeneous. Some CTCs exist as individual cells, some as clusters of tumor cells, some associated with blood cells such as platelets and many non-viable cells(64). Thus, the challenge for CTC research is thus not only to discriminate tumor cells circulating in the bloodstream from the huge majority of blood cells (14, 65), but to identify the subpopulation of CTCs that are truly representative of the primary tumor and those that are capable of forming the lethal metastases that are ultimately responsible for an adverse prognosis. Therefore, CTCs captured must be further characterized, in order to establish their origin and their genetic profile. Most of the available protocols are focused either on the nucleic acid content or on the protein level. In the past, nucleic acid based detection approaches were commonly used as a CTC detection method, which indeed are very sensitive techniques, relying on the detection of specific DNA or RNA sequences differentially expressed by tumor cells. However, in recent years there has been a preferential shift toward cytometric methods by which cells remain intact, hence morphology can be observed, cells can be enumerated and further analysis by immunofluorescence or immunohistochemical staining techniques such as fluorescent *in situ* hybridization (FISH) or even DNA/RNA extraction are practically or theoretically possible(36, 42, 66, 67).

Several currently available CTCs detection platforms were verified in various clinical settings (14, 16, 61). Clinical studies with CTCs have focused on enumerating CTCs in cohorts of patients and then comparing the number of CTCs to clinical characteristics. A correlation between CTC numbers and prognosis and progression of disease has been established in patients with metastatic breast, colon, prostate and lung cancers (19, 31-33, 41). Compared with enumeration of CTCs in metastatic disease, detection of CTCs in early stage cancer patients is more challenging, but there are some researches to suggest that CTC numbers may help predict prognosis in earlier stage patients(68, 69). Longitudinal monitoring of CTCs-derived genotypes may provide a noninvasive approach to identify drug-sensitivity and resistance-associated markers, guiding therapeutic decisions (70). In addition to their potential use in directing the treatment, CTCs may also hold the key to monitoring for early dissemination of cancer(44, 50). However, the different assays, alternative definition criteria for CTCs, different, screened populations, limited number of patients, and different stages of the disease make the clinical significance of CTCs detection difficult to interpret. Therefore, the specificity and clinical utility of these methods still have to be demonstrated in large prospective multicenter studies to reach the high level for introduction into clinical practice.

5. CLINICAL APPLICATION OF CTCS IN NSCLC

Because peripheral blood samples can be obtained routinely and more readily, detection of CTCs may be a better tool to evaluate prognosis and rapidly assess therapeutic response. CTCs has been shown to correlate with poor progression free survival (PFS) and overall survival (OS) in breast, colorectal, and prostate cancers (41). When comparing data detected with the same technology, incidence of CTCs in NSCLC was lower compared with other tumors(23, 71). However, high definition CTC (HD-CTC) technology, a non-enrichment based method, is not only more sensitive in lung cancer, but adds an ability to examine multiparametric characteristics of CTCs such as nuclear size, CK expression, clustering and other cell categories etc. The HD-CTC assay applied in NSCLC identifies CTCs in 73% of all patients, including 85% of patients with early stage disease and 79% of patients with locally advanced disease, making this assay more promising in NSCLC(70). Given the heterogeneity of NSCLC, no single marker gene was expressed in all NSCLC tumors and not in peripheral blood cells; therefore a multi-marker gene approach was employed. An assay, based on immunomagnetic bead enrichment followed by quantitative real-time PCR (QPCR) analyses of four selected marker genes, showed sufficient sensitivity and good specificity in discriminating CTCs in peripheral blood samples of advanced NSCLC patients from healthy controls(18). Another study showed that the complementary dual technology approach of CTC analysis, a marker-dependent (CellSearch) and a marker-independent (ISET), allows more complete exploration of CTCs in patients with NSCLC(72).

Thus, the progress in CTCs technology development increased the potential applicability of technology for clinical utility of lung cancer patients. Most of the generally used techniques for CTCs detection have been investigated in lung cancer model with some successes in diagnosis, monitoring, and treatment decision making in patients with NSCLC. In patients with NSCLC, there is evidence that numeration of CTCs is prognostic and correlated with overall survival and stage of disease, and that CTCs counted before and during treatment mirror treatment response(31). Higher numbers of detected CTCs were associated with an unfavorable prognosis(20). The detection of EpCAM/MUC1 mRNA-positive CTCs before and after surgery is useful for predicting a poor prognosis in NSCLC patients who undergo curative surgery(22). A study by ISET, found that 30% of all samples who underwent surgery at all stages of NSCLC were identified to be positive for CTCs, while patients with high level of CTCs turned out to have a worse prognosis(73). A propensity for increased CTC detection was observed as the disease progressed in individual patients(70). In patients with primary lung cancer, the detection of CTCs in the blood was also found to be a useful surrogate marker of distant metastasis(74)

Differential diagnosis of suspicious lung masses is essential for the selection of the appropriate therapy strategy. Increasingly, clinicians are relying on the genetic and molecular phenotype of certain cancers to guide treatment decisions. Some studies have investigated the molecular and genetic characterization of CTCs, beyond simple enumeration. CTCs offer promise as a surrogate for tissue where there is insufficient tissue for molecular analysis. With advances in technologies for examination of intact CTCs, personalized medicine with treatment selection according to molecular characteristics of CTCs was proposed. In patients with molecularly defined

subtypes of NSCLC, CTCs demonstrate the same molecular changes as the cancer cells of the tumor (24, 61, 62). Along with imaging and pathological diagnostic procedures, laboratory determinations of CTCs are essential for the histological differential diagnosis in lung cancer patients. A powerful example of the application of CTCs genotyping to personalized medicine lies in the detection of epidermal growth factor receptor (EGFR) mutations in patients with NSCLC(8), which is responsive to selective EGFR kinase inhibitors such as gefitinib and erlotinib(21, 75). EGFR mutation analysis in lung cancer is now standard parts of diagnostic tests used by clinicians. Evidence of a correlation between decreases in CTCs counts and radiographic response in patients with advanced NSCLC was evaluated, which suggest a potential role for using CTCs decreases as an early indication of response to therapy(8, 21). Correlation of low expression of ERCC1 on CTCs with longer PFS was observed in patients with metastatic NSCLC receiving platinum-based therapy (17). CTC counts are correlated with radiographic response and patient survival and to evaluate whether candidate predictive biomarkers can be assessed through molecular and cell–based assays on captured CTCs(21). In conclusion, progress in development of CTCs technology increased the potential applicability of CTCs detection in clinical utility for NSCLC patients to aid in both the earlier diagnosis and treatment management.

6. FUTURE DIRECTION

In contrast to tumor tissue or metastases, CTCs are easily obtained by venipuncture, thereby enabling repeated sampling over time. Nevertheless, due to the low number of these cells detectable with the currently available methods especially in patients with early-stage tumors, analysis of CTCs is still not part of routine tumor diagnostics in clinical practice. Given their rarity in the circulation, our knowledge of CTCs is heavily dependent on the technological approaches used for their detection and isolation. Most of studies on CTCs in NSCLC have some limitations, relatively small sample size and insufficiently the follow-up period. Therefore, the different technologies and also the differences among the screened populations make the clinical significance of CTCs detection difficult to interpret. In CTCs studies involving lung cancer, CTCs enumeration as a method to monitor patient progress has not been as convincing, because CTCs were not found in many study patients, and those patients with CTCs had numbers far lower than other cancer types. Improvements in CTCs capture efficiency, quantization, imaging, and molecular analyses would promote further clinical applications. In addition, we need to identify the most aggressive subset of CTCs that are the metastasis-initiating cells. Therefore, a prospective study with long-term follow-up in a large number of patients with NSCLC is required. Although extremely rare, CTCs based analysis enable us to study both quantitative and qualitative properties of the malignancy. Therefore, technological developments of sensitivity of CTCs detection may provide new opportunities for early diagnosis of metastasis, and unprecedented opportunity to develop therapeutic strategies to effectively treat and prevent NSCLC.

7. ACKNOWLEDGEMENT

Yi Han and Chongyu Su are the co-first authors.

8. REFERENCE

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J: Cancer statistics, 2008. *CA Cancer J Clin* 58:71-96 (2008)

2. Duffy MJ, McGowan PM, Gallagher WM: Cancer invasion and metastasis: changing views. *J Pathol* 214:283-293 (2008)

3. Wittekind C, Neid M: Cancer invasion and metastasis. *Oncology* 69 Suppl 1:14-16 (2005)

4. Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. *Cell* 144:646-674 (2011)

5. Chiang AC, Massague J: Molecular basis of metastasis. *N Engl J Med* 359:2814-2823 (2008)

6. Pantel K, Brakenhoff RH: Dissecting the metastatic cascade. *Nat Rev Cancer* 4:448-456 (2004)

7. Chambers AF, Groom AC, MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2:563-572 (2002)

8. Maheswaran S, Sequist LV, Nagrath S, Ulkus L: Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 359:366-377 (2008)

9. Aguirre-Ghiso JA: Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer* 7:834-846 (2007)

10. Pantel K, Alix-Panabieres C, Riethdorf S: Cancer micrometastases. *Nat Rev Clin Oncol* 6:339-351 (2009)

11. Aguirre-Ghiso JA: On the theory of tumor self-seeding: implications for metastasis progression in humans. *Breast Cancer Res* 12:304 (2010)

12. Maheswaran S, Haber DA: Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev* 20:96-99 (2010)

13. Alix-Panabieres C, Schwarzenbach H, Pantel K: Circulating tumor cells and circulating tumor DNA. *Annu Rev Med* 2012;63:199-215.

14. Sun YF, Yang XR, Zhou J: Circulating tumor cells: advances in detection methods, biological issues, and clinical relevance. *J Cancer Res Clin Oncol* 137:1151-1173 (2011)

15. Pantel K, Alix-Panabieres C: Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med* 16:398-406 (2010)

16. Bednarz-Knoll N, Alix-Panabieres C, Pantel K: Clinical relevance and biology of circulating tumor cells. *Breast Cancer Res* 13:228 (2011)

17. Das M, Riess JW, Frankel P, Schwartz E: ERCC1 expression in circulating tumor cells (CTCs) using a novel detection platform correlates with progression-free survival (PFS) in patients with metastatic non-small-cell lung cancer (NSCLC) receiving platinum chemotherapy. *Lung Cancer* 77:421-426 (2012)

18. Devriese LA, Bosma AJ, van de Heuvel MM, Heemsbergen W: Circulating tumor cell detection in advanced non-small cell lung cancer patients by multi-marker QPCR analysis. *Lung Cancer* 75:242-247 (2012)

19. Wendel M, Bazhenova L, Boshuizen R, Kolatkar A, Honnatti M: Fluid biopsy for circulating tumor cell identification in patients with early-and late-stage non-small cell lung cancer: a glimpse into lung cancer biology. *Phys Biol* 9:016005 (2012)

20. Hofman V, Bonnetaud C, Ilie MI, Vielh P, Vignaud JM: Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker. *Clin Cancer Res* 17:827-835 (2011)

21. Punnoose EA, Atwal S, Liu W, Raja R, Fine BM: Evaluation of circulating tumor cells and circulating tumor DNA in non-small cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. *Clin Cancer Res* 18:2391-2401 (2012)

22. Zhu WF, Li J, Yu LC, Wu Y, Tang XP: Prognostic value of EpCAM/MUC1 mRNA-positive cells in non-small cell lung cancer patients. *Tumour Biol* (2013)

23. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 10:6897-6904 (2004)

24. Van't Westeinde SC, van Klaveren RJ: Screening and early detection of lung cancer. *Cancer J* 17:3-10 (2011)

25. Molina R, Holdenrieder S, Auge JM, Schalhorn A: Diagnostic relevance of circulating biomarkers in patients with lung cancer. *Cancer Biomark* 6:163-178 (2010)

26. Herbst RS, Heymach JV, Lippman SM: Lung cancer. *N Engl J Med* 359:1367-1380 (2008)

27. Tuveson DA, Jacks T: Modeling human lung cancer in mice: similarities and shortcomings. *Oncogene* 18:5318-5324 (1999)

28. Gadgeel SM: New targets in non-small cell lung cancer. *Curr Oncol Rep* 15:411-423 (2013)

29. Spira A, Ettinger DS: Multidisciplinary management of lung cancer. *N Engl J Med* 350:379-392 (2004)

30. Tsuboi M, Ohira T, Saji H, Miyajima K, Kajiwara N: The present status of postoperative adjuvant chemotherapy for completely resected non-small cell lung cancer. *Ann Thorac Cardiovasc Surg* 13:73-77 (2007)

31. Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM: Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. *J Clin Oncol* 29:1556-1563 (2011)

32. Sastre J, Maestro ML, Puente J, Veganzones S, Alfonso R: Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. *Ann Oncol* 19:935-938 (2008)

33. Cristofanilli M: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *Semin Oncol* 33:S9-14 (2006)

34. Paget S: The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev* 8:98-101 (1989)

35. Krebs MG, Hou JM, Ward TH, Blackhall FH, Dive C: Circulating tumour cells: their utility in cancer management and predicting outcomes. *Ther Adv Med Oncol* 2:351-365 (2010)

36. Pantel K, Brakenhoff RH, Brandt B: Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer* 8:329-340 (2008)

37. Kim MY, Oskarsson T, Acharyya S, Nguyen DX: Tumor self-seeding by circulating cancer cells. *Cell* 139:1315-1326 (2009)

38. Pantel K, Alix-Panabieres C: Real-time Liquid Biopsy in Cancer Patients: Fact or Fiction? *Cancer Res* 73:6384-6388 (2013)

39. Hou JM, Greystoke A, Lancashire L, Cummings J: Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. *Am J Pathol* 175:808-816 (2009)

40. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD: Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 26:3213-3221 (2008)

41. Marrinucci D, Bethel K, Kolatkar A, Luttgen MS: Fluid biopsy in patients with metastatic prostate, pancreatic and breast cancers. *Phys Biol* 9:016003 (2012)

42. Paris PL, Kobayashi Y, Zhao Q, Zeng W, Sridharan S: Functional phenotyping and genotyping of circulating tumor cells from patients with castration resistant prostate cancer. *Cancer Lett* 277:164-173 (2009)

43. Allan AL, Keeney M: Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. *J Oncol* 2010:426218 (2010).

44. Sleijfer S, Gratama JW, Sieuwerts AM, Kraan J: Circulating tumour cell detection on its way to routine diagnostic implementation? *Eur J Cancer* 43:2645-2650 (2007)

45. Paterlini-Brechot P, Benali NL: Circulating tumor cells (CTC) detection: clinical impact and future directions. *Cancer Lett* 253:180-204 (2007)

46. Tibbe AG, Miller MC, Terstappen LW: Statistical considerations for enumeration of circulating tumor cells. *Cytometry A* 71:154-162 (2007)

47. Pinzani P, Salvadori B, Simi L, Bianchi S, Distante V: Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecular analysis by laser microdissection. *Hum Pathol* 37:711-718 (2006)

48. Vona G, Sabile A, Louha M, Sitruk V, Romana S: Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells. *Am J Pathol* 156:57-63 (2000)

49. Gertler R, Rosenberg R, Fuehrer K, Dahm M, Nekarda H, Siewert JR: Detection of circulating tumor cells in blood using an optimized density gradient centrifugation. *Recent Results Cancer Res* 162:149-155 (2003)

50. Bischoff J, Rosenberg R, Dahm M, Janni W, Gutschow K: Minimal residual disease in bone marrow and peripheral blood of patients with metastatic breast cancer. *Recent Results Cancer Res* 162:135-140 (2003)

51. Rosenberg R, Gertler R, Friederichs J, Fuehrer K, Dahm M: Comparison of two density gradient centrifugation systems for the enrichment of disseminated tumor cells in blood. *Cytometry* 49:150-158 (2002)

52. Zheng S, Lin HK, Lu B, Williams A, Datar R: 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. *Biomed Microdevices* 13:203-213 (2011)

53. Somlo G, Lau SK, Frankel P, Hsieh HB, Liu X: Multiple biomarker expression on circulating tumor cells in comparison to tumor tissues from primary and metastatic sites in patients with locally advanced/inflammatory, and stage IV breast cancer, using a novel detection technology. *Breast Cancer Res Treat* 128:155-163 (2011)

54. Andreopoulou E, Yang LY, Rangel KM, Reuben JM, Hsu L: Comparison of assay methods for detection of circulating tumor cells in metastatic breast cancer: AdnaGen AdnaTest BreastCancer Select/Detect versus Veridex CellSearch system. *Int J Cancer* 130:1590-1597 (2012)

55. Van der Auwera I, Peeters D, Benoy IH, Elst HJ, Van Laere SJ: Circulating tumour cell detection: a direct comparison between the CellSearch System, the AdnaTest and CK-19/mammaglobin RT-PCR in patients with metastatic breast cancer. *Br J Cancer* 102:276-284 (2010)

56. Gorges TM, Tinhofer I, Drosch M, Rose L, Zollner TM: Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer* 12:178 (2012)

57. Bonnomet A, Brysse A, Tachsidis A, Waltham M, Thompson EW: Epithelial-to-mesenchymal transitions and circulating tumor cells. *J Mammary Gland Biol Neoplasia* 15:261-273 (2010)

58. Christiansen JJ, Rajasekaran AK: Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* 66:8319-8326 (2006)

59. van de Stolpe A, Pantel K, Sleijfer S, Terstappen LW, den Toonder JM: Circulating tumor cell isolation and diagnostics: toward routine clinical use. *Cancer Res* 71:5955-5960 (2011)

60. Mikolajczyk SD, Millar LS, Tsinberg P, Coutts SM: Detection of EpCAM-Negative and Cytokeratin-Negative Circulating Tumor Cells in Peripheral Blood. *J Oncol* 2011:252361 (2011)

61. Parkinson DR, Dracopoli N, Petty BG, Compton C, Cristofanilli M: Considerations in the development of circulating tumor cell technology for clinical use. *J Transl Med* 10:138 (2012)

62. Fusi A, Metcalf R, Krebs M, Dive C, Blackhall F: Clinical Utility of Circulating Tumour Cell Detection in Non-Small-Cell Lung Cancer. *Curr Treat Options Oncol* (2013)

63. Marrinucci D, Bethel K, Luttgen M, Bruce RH: Circulating tumor cells from well-differentiated lung adenocarcinoma retain cytomorphologic features of primary tumor type. *Arch Pathol Lab Med* 133:1468-1471 (2009)

64. Cho EH, Wendel M, Luttgen M, Yoshioka C, Marrinucci D: Characterization of circulating tumor cell aggregates identified in patients with epithelial tumors. *Phys Biol* 9:016001 (2012)

65. Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H: Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. *Clin Cancer Res*;8:2073-2084 (2002)

66. Watson MA, Ylagan LR, Trinkaus KM, Gillanders WE, Naughton MJ: Isolation and molecular profiling of bone marrow micrometastases identifies TWIST1 as a marker of early tumor relapse in breast cancer patients. *Clin Cancer Res* 13:5001-5009 (2007)

67. Smirnov DA, Zweitzig DR, Foulk BW, Miller MC: Global gene expression profiling of circulating tumor cells. *Cancer Res* 65:4993-4997 (2005)

68. Molloy TJ, Devriese LA, Helgason HH, Bosma AJ, Hauptmann M: A multimarker QPCR-based platform for the detection of circulating tumour cells in patients with early-stage breast cancer. *Br J Cancer* 104:1913-1919 (2011)

69. Graves H, Czerniecki BJ: Circulating tumor cells in breast cancer patients: an evolving role in patient prognosis and disease progression. *Patholog Res Int* 2011:621090 (2011)

70. Nieva J, Wendel M, Luttgen MS, Marrinucci D, Bazhenova L: High-definition imaging of circulating tumor cells and associated cellular events in non-small cell lung cancer patients: a longitudinal analysis. *Phys Biol* 9:016004 (2012)

71. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D: Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 450:1235-1239 (2007)

72. Krebs MG, Hou JM, Sloane R, Lancashire L, Priest L: Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. *J Thorac Oncol* 7:306-315 (2012)

73. Boshuizen R, Kuhn P, van den Heuvel M: Circulating tumor cells in non-small cell lung carcinoma. *J Thorac Dis* 4:456-458 (2012)

74. Tanaka F, Yoneda K, Kondo N, Hashimoto M, Takuwa T: Circulating tumor cell as a diagnostic marker in primary lung cancer. *Clin Cancer Res* 15:6980-6986 (2009)

75. Sequist LV, Bell DW, Lynch TJ, Haber DA: Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer: *J Clin Oncol* 25:587-595 (2007)

Abbreviations: CTCs: Circulating tumor cells; NSCLC: Non-small cell lung cancer; DTCs: disseminated tumor cells; ISET: Isolation by Size of Epithelial Tumor Cells; EpCAM: Epithelial cell adhesion molecule; FISH: fluorescent *in situ* hybridization; HD-CTC: high definition CTC; QPCR: quantitative real-time PCR

Key Words: CTCs, Non-Small-Cell Lung Cancer, Prognosis, Tumor, Patient, Tumor Marker, Clinical, Metastasis, Overall Survival, Therapy, Diagnosis, Review

Send correspondence to: Zhidong Liu, Department of Thoracic Surgery II, Beijing Chest Hospital, Capital Medical University, No. 97 Ma Chang, Tongzhou District, Beijing, 100049 , China, Tel: 86-13601338599, Fax: 86-10-89509306, E-mail: lzdzrd@yeah.net