Circulating tumor cells as lung cancer biomarkers

Maria P Wong

Department of Pathology, The University of Hong Kong, Hong Kong

J Thorac Dis 2012;4(6):631-634. DOI: 10.3978/j.issn.2072-1439.2012.10.05

Circulating tumor cells (CTC) were first observed under light microscopy in 1869 by the Australian physician Thomas Ashworth, who found tumor cells with "similar appearances as the primary tumor" in the circulation of a man with metastatic cancer (1). This important discovery identified the haematogenous route as a medium of distant cancer spread. With recent technological advances, scientists are now able to detect, characterize and isolate CTC, providing a valuable handle for studying the biology of tumor metastasis and a potential tool for guiding clinical cancer management.

Technology platforms for CTC detection

CTC are considered as rare cells compared to the number of circulating leukocytes. Even in advanced cancers with large numbers of detectable CTC, leukocytes are still in the order of 10^4 to 10^7 folds more abundant. The detection of cancer-related molecules expressed on Ficoll-extracted peripheral blood nucleated cells by sensitive methods such as RT-PCR has been used to infer the presence of CTC but this approach is indirect; non-specific expression by leukocytes cannot be distinguished. On the other hand, despite successful vascular entry, not all CTC have equal biological potentials in seeding tumor metastasis. Heterogeneity in their intrinsic viability and functional capacities are likely to affect whether CTC would be able to persevere in the adverse intravascular environment, survive coagulation, innate or immune defense, and interact productively with foreign tissues to establish distant metastasis. In essence, these phenotypic differences would affect whether CTC would be useful as a surrogate endpoint biomarker and its eligibility as a clinical tool for cancer prognostication. To enable a more direct analysis of CTC properties and significance, the initial step involves laboratory procedures to capture and concentrate the relatively rare CTC from whole blood. Two main principles have been employed based on either immuno-selection in a fluid flow chamber or size separation by a filtration device.

Cell Search system (CS)

The Cell Search™ System (Veridex) is the pioneer in developing a standardized commercial system for CTC detection. A colloidal ferrofluid containing magnetic nanoparticles coated with anti-EpCAM antibodies against epithelial antigens is mixed with a sample of whole blood for immunomagnetic separation. The epithelial cells-enriched sample is then fixed and stained with fluorescence-labeled antibodies against a panel of cytokeratins (CK), the leukocyte common antigen CD45, and counter-stained with the fluorescence DNA dye 4',6-diamidino-2-phenylindole (DAPI). Cells displaying a profile of $DAPI^+/CK^+/CD45^-$ are selected as CTC. The system is semi-automated and requires a final step of manual confirmation of the fluorescence cell images by trained personnel. Currently, the system has gained approval from the U.S. Food and Drug Administration (FDA) for providing CTC enumeration tests in a clinical setting. In advanced breast cancers, pre-treatment baseline counts of ≥5 CTC per 7.5 mL whole blood is statistically associated with shorter progression free survival (PFS) and overall survival (OS). Similarly, baseline counts of ≥5 and ≥3 CTC are approved as surrogate prognostic markers in metastatic prostate and colon cancer patients, respectively.

Importantly, false positive results have been repeatedly reported in patients with a wide range of non-malignant and inflammatory conditions (2). For example, up to 37 cells per blood sample satisfying the selection criteria of CTC have been found in patients with diverticulosis and in 11.5% of patients with inflammatory bowel diseases (3). Therefore, this method has limited specificity as an initial test for cancer screening and long term follow up is required to clarify whether these individuals harbour a subclinical cancer. The role of the Cell Search (CS) system in evaluating CTC for cancer management must be clearly defined and the test must be applied only to the specific clinical situation for which the system is approved for use.

Isolation by size of epithelial tumor cells (ISET)

Cancer cells are generally larger than normal cells and this feature has been exploited as a primary CTC selection criterion without

Corresponding to: Maria P Wong. Department of Pathology, Queen Mary Hospital, The University of Hong Kong, Pokfulam Road, Hong Kong. Email: mwpik@hku.hk.

Submitted Sep 11, 2012. Accepted for publication Oct 15, 2012.
Available at www.jthoracdis.com

ISSN: 2072-1439

involving immuno-labeling of cell surface markers. The tumor cells of even small cell lung cancer (SCLC) are larger than circulating lymphocytes (1.5× to 4× the size of small lymphocytes) or large monocytes (mean diameter about 7.2 μm) (4). Two devices are currently available. The Rarecells system captures CTC by drawing a volume of diluted whole blood through a microporous filter with average pore size of 8 μm while the ScreenCell uses a filter of 7.5 μm average pore size. Both systems can capture CTC as individual cells and as cell clusters or microemboli with a claimed sensitivity of 1 CTC per mL whole blood. It also offers the additional advantage that captured cells are viable which can be harvested for culture or animal studies.

There is no standardized automated method for subsequent assessment of the retained cells and different inclusion criteria with or without further immunophenotypic characterization have been adopted in different studies. In a study of 808 samples from healthy subjects and patients with mixed cancers or benign conditions, 10 cytopathologists from the same institution employing calibrated cell size (3 times that of the micropore size or 24 μm) and classical cyto-morphological features such as nuclear pleomorphism as diagnostic criteria were able to achieve a low inter-observer variability. However, 5.3% of patients with non-malignant conditions yielded positive results (5), indicating reactive cells of unknown identity but displaying morphologically malignant features are present in the circulation. The data infer in cancer patients, cells arising from reactive tissues surrounding the tumor might also be falsely counted as positive cells. Such false positivity indicates further ancillary tests such as molecular or immuno-phenotyping should be used for ascertaining the malignant identity of cells selected by ISET.

Microfluidics devices

Various microfluidics systems utilizing different capture principles such as tumor cell size and deformability have been investigated to enrich and identify CTC from whole blood (6). Notably, a promising system designated as the CTC-chip in which anti-EpCAM antibodies are coated on 78,000 microposts sandwiched within the microfluidics chamber is under active investigation (7). The CTC are captured by immuno-binding as blood is gently pumped through the chamber, and capture efficiency is further enhanced by the device's "herring-bone" modification in which microvortices are created in the path of blood flow by the special microposts distribution. Similar to ISET, the captured live cells can be harvested for functional studies. Pilot studies on different cancers have shown a higher CTC yield and superior detection sensitivity of almost 100% in clinical samples (7).

Clinical utility of CTC detection

The clinical value of CTC as a surrogate biomarker depends on how consistently and accurately CTC can reflect tumor load, prognosis and response to therapy. If CTC enumeration could stratify patients into prognostic subgroups with differential outcomes, then treatment plans could be modified to alter the course of the cancer and strike an impact on cancer management. On the other hand, the CTC analysis platforms and protocols should be standardized to enable quantitative, robust and reproducible data to be collected and validated across different laboratories. Scalability of the system for high throughput performances is also an important consideration in clinical application.

Optimal platform and standardization of protocol

Both the immune selection and size separation approaches have been utilized for CTC detection in lung cancer. The CS and ISET systems have major differences in requirements for budget, laboratory space and technical expertise. The performance efficiency and throughput are also important issues affecting the decision on which system to adopt in busy clinical oncology practices. In resectable NSCLC, CTC are detected in 19% to 39% of patients by CS analysis, and in 36% to 50% by the ISET approach (8,9). Thus, the relatively low sensitivity levels are unlikely to justify the use of CTC analysis as a routine diagnostic screen for the detection of early lung cancers. In metastatic NSCLC, CTC counts are generally higher and investigations have found 32% to 78% positivity by CS and up to 80% by ISET (8,10). Data from different laboratories have repeatedly shown limited consistency of results obtained by the CS and ISET approaches. At least 2 studies directly comparing CS and ISET in the same patient cohort have shown low concordance rates. In a study of 210 mixed types of resectable NSCLC with an overall pre-operative detection rate of 69%, only 20% of cases were detected by both CS and ISET, while 19% were detectable by CS only and 30% by ISET only (8). There was no significant correlation between cases detected by the two methods indicating different cell subpopulations are being measured. Further, the investigators observed amongst cases with morphologically malignant cells detected by ISET, only 39% contained CTC expressing cytokeratins while 11% showed vimentin expression only without cytokeratin. Similarly, Krebs *et al*. reported in 40 patients with stage III and IV NSCLC, pretreatment CTC were found in 23% of patients by CS and 80% by ISET, with some EpCAM$^-$ cells included in the ISET-selected cases (10). The cytokeratin$^-$/vimentin$^+$ cells are interpreted as most likely representing cells which have lost epithelial cell adhesion molecules and acquired stromal differentiation features during the process of epithelial-mesenchymal transition. Analysis methods that use initial selection by the anti-EpCAM antibody would have missed these cells leading to a falsely low CTC detection rate. However, CTC-chip which also yields CTC

after EpCAM pre-selection has shown a much higher detection rate of up to 100% in metastatic NSCLC. The contrasting results are speculated to be related to significant loss of CTC during the complex sample preparation procedure required by CS which can be avoided by the one-step protocol of CTC-chip.

CTC threshold counts and role as a surrogate biomarker

It is important to understand the performance of CTC as a cancer biomarker in terms of its sensitivity and specificity. A low sensitivity might lead to under- or delayed treatment but low specificity and false positive calls might lead to needless stress, over-investigation or even unnecessary treatment of patients. These are most important for early or subclinical cases before a firm diagnosis of cancer is established. Tanaka *et al*. investigated CTC counts by CS in a cohort of 150 patients suspected to have lung cancer. Using one CTC as the threshold count, the sensitivity and specificity for diagnosing lung cancer was 30.4% and 88.0%, respectively. Importantly, in 25 patients subsequently found to have a non-malignant lung lesion, 1 to 2 CTC were found in 3 (12%) patients, indicating a cutoff value of 2 or above is required to avoid false positive calls when CS is used to discriminate between lung cancer and reactive lung conditions. On the other hand, in 250 resectable NSCLC patients analyzed by ISET, 2% showed morphologically benign cells and 6% displayed indeterminate features in their preoperative samples (11). Morphologically low grade tumor cells have been detected in the circulation of patients with well differentiated carcinomas. If diagnostic criteria are too stringent, these CTC would be counted as false negatives due to their lack of overt malignant morphology. Overall, as in all laboratory tests, the sensitivity and specificity are reciprocally related. A carefully balanced cutoff value based on sound diagnostic criteria, sufficient and validated clinical data are required to enable CTC analysis to benefit the maximum number of patients.

The utility of CTC as a surrogate biomarker has been most widely studied in advanced lung cancer patients. For example, in 101 stage III and IV patients analyzed by CS, Krebs *et al*. observed when the baseline cutoff count of 5 CTC was adopted, they were able to dichotomize patients into those with statistically different progression-free survival (PFS) and overall survival (OS) independent of stage and performance status (12). Patients with <5 CTC per 7.5 mL blood had a mean PFS of 6.8 months, OS of 8.1 months and 29% death rate within 6 months while those with ≥5 CTC showed PFS of 2.4 months, OS of 4.3 months and 93% death rate in 6 months. Other investigators have also reported a prognostic value of CTC counts but many studies using CS have adopted a cutoff threshold of 1 or 2 CTC per sample (8). This threshold overlaps with the number of false positive cells observed in healthy controls, hence, it is difficult to evaluate the true significance of these results.

On the other hand, CTC are detected more frequently and in higher numbers in patients with SCLC. Counts of >1,000 per sample have been observed by CS in 70% to 85% of pretreatment patients (13). Naito *et al*. systematically evaluated the hazard ratios of a range of CTC levels and using 8 CTC for stratification, the initial count, drop in CTC after one chemotherapy cycle and post-treatment CTC levels have been reported as independent prognostic indicators for OS (14). However, as for NSCLC, other threshold values have been adopted in other studies, and more data are needed to determine a consensus cutoff level (13).

Mutation detection biomarker predicting target drug sensitivity

Somatic mutations of cancer genes can act as excellent markers since they are cancer cell-specific and such genomic changes are not affected by changes in protein marker expression due to phenotypic adaptation of tumor cells to different micro-environments. Mutations identified in circulating cells which are identical to those of the primary tumors provide concrete proof that the cells being detected are CTC. This application is particularly important in view of the changing trend favoring personalized medicine against known driver oncogenes. CTC abundance may also closely reflect the patient's immediate condition at the cellular and molecular levels, providing much more sensitive and timely information such as response to targeted therapy, early tumor recurrence or even detection of resistant mutations compared to traditional radiological criteria. These applications and utility in cases with EGFR mutations have been demonstrated by different studies using CS approach (15,16). Initial data also show this approach might have a better predictive power than mutation detection in circulating DNA, probably because such DNA released from the tumor is often fragmented and likely to reflect degenerating or necrotic tumor cells. Furthermore, certain molecular tests such as fluorescence in-situ hybridization (FISH) required for the detection of lung cancer fusion genes such as *-ALK* is feasible on intact CTC but not circulating DNA (17). Overall, the comparative usefulness of circulating DNA and CTC in lung cancer management remains to be verified.

Challenges and opportunities of CTC detection

Studies have shown CTC detection and characterization hold promising values. However, these data are derived from a variety of evaluation methods and protocols, and used to address different clinical endpoints, making it difficult to compare results and to draw meaningful conclusions. To derive universally applicable guidelines for clinical management, some major hurdles need to be overcome.

The most important issue concerns the sensitivity of CTC detection. A high sensitivity not only increases the number of

potential patients who would benefit from this test, it should also yield a higher CTC count per sample. This is important for demonstrating a significant difference from the baseline false positive counts in non-malignant conditions and thus improving the power of CTC to discriminate cancer from reactive changes.

While evaluation of merely the cytokeratin and mesenchymal markers have already revealed CTC heterogeneity, it is highly likely that more refined phenotypic characterization would help to decipher the complex mechanisms regulating distant metastasis and in turn, patient outcome. With the ever reducing costs of high throughput, genome-wide profiling facilities such as next generation sequencing, CTC can be harvested for comparison of different disease milestones such as primary and metastatic, pre- and post-treatment, drug-sensitive and resistant cancers. Until the crucial pathways or biomarkers are identified, these studies would require a large number of CTC for phenotyping and functional experiments. A highly sensitive CTC detection and isolation system is necessary to provide such samples and avoid over-drawing of blood from patients.

Lastly, it is imperative that clinicians and patients understand the prognostic and predictive implications of CTC assessment in terms of recurrence risks, treatment response, survival probabilities and durations. The values and limitations of a single CTC test performed at the start or end of a treatment course might have different meanings, and the usefulness of serial tests for cancer surveillance must be clarified. Currently, the data accumulated from different studies are insufficient to provide verified data for information or contribute to the treatment decision process. Data from large scale, multi-institutional and well coordinated studies with proper controls, clearly defined protocols and endpoints are needed to advance the clinical utility of CTC assessment.

Acknowledgements

Disclosure: The author declares no conflict of interest.

References

1. Ashworth TR. A case of cancer in which cells similar to those in the tumors were seen in the blood after death. Aus Med J 1869;14:146-9.
2. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004;10:6897-904.
3. Pantel K, Denève E, Nocca D, et al. Circulating epithelial cells in patients with benign colon diseases. Clin Chem 2012;58:936-40.
4. Yasaka T, Mantich NM, Boxer LA, et al. Functions of human monocyte and lymphocyte subsets obtained by countercurrent centrifugal elutriation: differing functional capacities of human monocyte subsets. J Immunol 1981;127:1515-8.
5. Hofman VJ, Ilie MI, Bonnetaud C, et al. Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method: promises and pitfalls. Am J Clin Pathol 2011;135:146-56.
6. Tan SJ, Lakshmi RL, Chen P, et al. Versatile label free biochip for the detection of circulating tumor cells from peripheral blood in cancer patients. Biosens Bioelectron 2010;26:1701-5.
7. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235-9.
8. Hofman V, Ilie MI, Long E, et al. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay™ and the isolation by size of epithelial tumor cell method. Int J Cancer 2011;129:1651-60.
9. Tanaka F, Yoneda K, Kondo N, et al. Circulating tumor cell as a diagnostic marker in primary lung cancer. Clin Cancer Res 2009;15:6980-6.
10. Krebs MG, Hou JM, Sloane R, et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol 2012;7:306-15.
11. Hofman V, Long E, Ilie M, et al. Morphological analysis of circulating tumour cells in patients undergoing surgery for non-small cell lung carcinoma using the isolation by size of epithelial tumour cell (ISET) method. Cytopathology 2012;23:30-8.
12. Krebs MG, Sloane R, Priest L, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. J Clin Oncol 2011;29:1556-63.
13. Hou JM, Krebs MG, Lancashire L, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol 2012;30:525-32.
14. Han K, Cao W, Che J, et al. First line chemotherapy with weekly docetaxel and cisplatin in elderly patients with advanced non-small cell lung cancer: a multicenter phase II study. J Thorac Oncol 2009;4:512-7.
15. Punnoose EA, Atwal S, Liu W, et al. 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 2012;18:2391-401.
16. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008;359:366-77.
17. Ilie M, Long E, Butori C, et al. ALK-gene rearrangement: a comparative analysis on circulating tumour cells and tumour tissue from patients with lung adenocarcinoma. Ann Oncol 2012;23:2907-13.

Cite this article as: Wong MP. Circulating tumor cells as lung cancer biomarkers. J Thorac Dis 2012;4(6):631-634. DOI: 10.3978/j.issn.2072-1439.2012.10.05