Review Article on Circulating Tumor Cells

Circulating tumor cell isolation: the assets of filtration methods with polycarbonate track-etched filters

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Abstract: Circulating tumor cells (CTCs) arise from primary or secondary tumors and enter the bloodstream by active or passive intravasation. Given the low number of CTCs, enrichment is necessary for detection. Filtration methods are based on selection of CTCs by size using a filter with 6.5 to 8 µm pores. After coloration, collected CTCs are evaluated according to morphological criteria. Immunophenotyping and fluorescence *in situ* hybridization techniques may be used. Selected CTCs can also be cultivated *in vitro* to provide more material. Analysis of genomic mutations is difficult because it requires adapted techniques due to limited DNA materials. Filtration-selected CTCs have shown prognostic value in many studies but multicentric validating trials are mandatory to strengthen this assessment. Other clinical applications are promising such as follow-up, therapy response prediction and diagnosis. Microfluidic emerging systems could optimize filtration-selected CTCs by increasing selection accuracy.

Keywords: Circulating tumor cells (CTCs); biological markers; filtration

Submitted Apr 23, 2015. Accepted for publication Aug 17, 2015.
doi: 10.3978/j.issn.1000-9604.2015.09.01
View this article at: http://dx.doi.org/10.3978/j.issn.1000-9604.2015.09.01

Introduction

Circulating tumor cells (CTCs) arise from primary or secondary tumors and enter the bloodstream by active or passive intravasation (1). Some of these cells may be responsible for metastasis after gaining properties of extravasation, survival and proliferation in the target tissue. Given their low frequency in the blood, 1 CTC/10^6-10^8 hematopoietic cells (2), the identification of CTCs must be preceded by enrichment. The two most common enrichment techniques are filtration method and immunomagnetic antigen-dependent technique. The latter selects CTCs by expression of membrane proteins. Each technique isolates different subgroups of CTC related to their heterogeneity (3).

Filtration methods are based on physical properties that allow isolation of CTCs by size. Indeed, CTCs are generally larger in size than hematopoietic cells, so most of these cells pass through the filter whereas CTCs are retained. Since the first demonstration of CTC isolation by filtration in 1964 (4), filtration devices have been improved and many downstream applications have been developed. In this review, we present only commercially available methods using polycarbonate track-etched filters. We develop the pros and cons of this technique compared to other enrichment methods. Furthermore, we review applications and the clinical utility of CTC filtration method.

Technique for cytomorphological analysis

Three filtration devices are commercially available (*Table 1*). For cytology, the most frequently used filtration methods are performed using Screencell®Cyto and ISET® devices, with track-etched polycarbonate filter. The filter pores measure 7.5 to 8 µm in diameter and retain 85-100% of tumor cells and only 0.1% of common blood cells (5).

The blood sample, collected in EDTA tube, is stored at 4 ℃ maximum 4 hours before filtration.

In Screencell®Cyto device, 3 mL of peripheral blood

Table 1 Commercially available filtration devices

Company	Device	Size of pores (µm)	Volume of peripheral blood (mL)	Motor of filtration	Main download applications
Screencell®	Screencell®Cyto	7.5	3	Aspiration created by a vacuum tube collector	Cytology, immunohistochemistry
	Screencell®MB	6.5	6	Aspiration created by a vacuum tube collector	Molecular biology
	Screencell®CC	6.5	6	Aspiration created by a vacuum tube collector	Culture
Rarecells®	ISET®	8	10 (10 spots)	Aspiration created by an electric vacuum pump	Cytology, immunohistochemistry, molecular biology, culture
Metacell®		8	10 to 50	Capillary action of the absorbent in contact with the membrane filter	Culture, and then cytology, immunohistochemistry, molecular biology

sample is diluted in 4 mL of filtration buffer mainly composed of saponin and paraformaldehyde (the patented formula is protected) during 8 minutes at room temperature. The 7 mL diluted sample is then filtrated in a unique filtration column by aspiration created by a vacuum tube collector. The device used is disposable (6).

In ISET® device commercialized by Rarecell®, peripheral blood is diluted 1:10 in the filtration buffer (0.175% saponin, 0.2% paraformaldehyde, 0.0372% EDTA, and 0.1% bovine serum albumin) for 10 minutes at room temperature. The module of filtration has 12 wells, enabling filtration of 12 samples of 10 mL of diluted solution. Each well includes a 0.6-cm-diameter surface filter. Filtration is initiated by aspiration created by a vacuum pump (7).

After blood filtration by Screencell®Cyto or RareCell® systems, the filtration membrane is disassembled from the module allowing staining or other techniques.

Hematoxylin-eosin or May-Grunwald-Giemsa stain may be performed on the filters. After staining and glass mounting, morphological analysis is carried out using routine optical microscope. CTC malignancy is identified using morphologic cytopathology criteria.

An alternative size-based separation technique is based on filtration driven by capillary-action (8). Metacell® have develop a commercially device using this technique for CTC culture which allows cytological identification. This device is presented in detail in the culture cell paragraph.

Advantages and disadvantages

CTC isolation by size method is fast, simple and reliable: filtration and coloration is an easy process and cytopathologists can identify CTCs using classic cytopathologic criteria (*Figure 1*). No complex instruments or adapted staff training is needed, but the help of a trained cytopathologist is mandatory to analyze the stained filters. A technical imperative however is the treatment of the sample within 4 hours of collection. An infrequent technical complication is characterized by blood clogging on the filter which stops filtration.

The enrichment device selects tumor cells on size criteria. Conversely, most PCR-based methods, immuno-magnetic techniques such as CellSearch® or other label-dependent microfluidic techniques retain only EpCAM positive cells. It is well know that tumor cells undergo epithelial-mesenchymal transition phenomena during tumorigenesis which enhances the invasive potential of the tumor cell (9). During this process, tumor cells lose the expression of epithelial markers and can acquire a mesenchymal or a stem-cell like phenotype (10). This population of tumor cells, missed by CellSearch® (11), seems to be critical in metastasis initiation (9). Another hypothesis of metastasis initiation is the invasion of target tissue by microemboli (12), which is well analyzed by filtration systems, contrary to CellSearch® (13). Indeed, these oligoclonal clusters reach a 23- to 50-fold increased metastatic potential compared to an isolated tumor cell in breast cancer (12).

Very rare cells, smaller than 8 µm, could be missed by filtration techniques. For example, in breast cancer, the size of CTC detected with CellSearch® system is comprised between 4 to 18 µm and obviously above 8-30 µm with filtration devices (13).

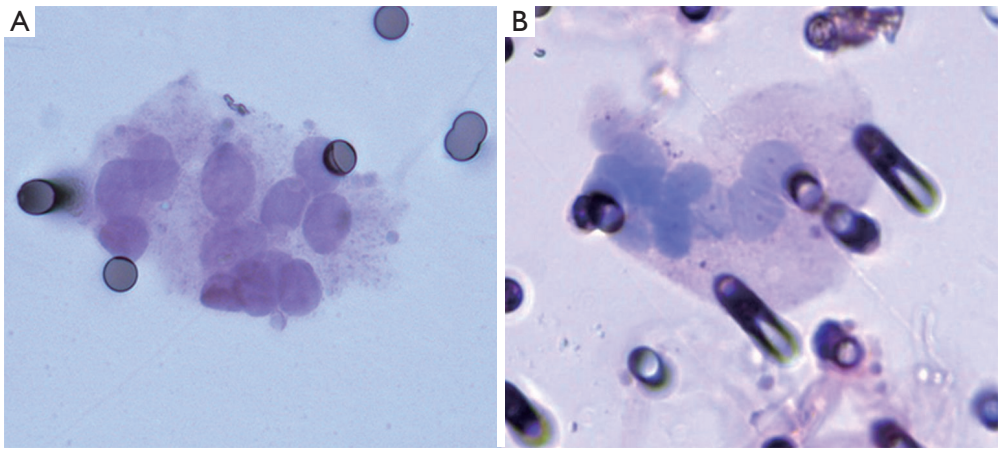

Figure 1 Clusters of circulating tumor cells (CTCs) with anisocaryosis, large nuclei and conspicuous nucleoli (Obj. × 40).

ISET® detects one tumor cell in 1 mL of peripheral blood (7,14). Several studies have compared the sensitivity of ISET® and CellSearch®. Farace *et al*. insisted on the superiority of ISET® compared to Cellsearch® in a series of 60 metastatic breast, prostate and lung cancer patients with 57 *vs*. 42 positive cases using ISET® and Cellsearch® respectively (15). A higher sensitivity of ISET® was demonstrated in hepatocellular carcinoma and in pancreatic cancer by Khoja *et al*. (16,17), in non-small cell lung cancer by Krebs *et al*. (13) and Hofman *et al*. (18). However, in these studies, CTCs were only detected in some patients by Cellsearch® and not by ISET®, probably corresponding to small CTCs.

Filtration devices allow selection of CTC on morphologic malignant criteria. Unlike this device, Cellsearch® defines CTC on immunological profile (EpCAM + cytokeratin + and CD45–) so benign epithelial cells, corresponding to these criteria, sometimes present in the blood stream could be misdiagnosed as CTCs. Therefore some false positive cases have been reported using this immunomagnetic technique (19).

False positivity also exists with filtration device due to the lack of specificity of this enrichment technique. Normal epidermal cells, which are collected by intra-dermic needle, can be present on the filter and misinterpreted as CTCs (they can be recognized with their large size and picnotic nuclei). Endothelial cells or rare hematologic cells such as megacaryocytes may also be present on the filter but are identified by pathologists according to their round and pale nucleus.

In some patients with no malignancy, circulating cells arising from parathyroid or thyroid adenoma have been described and can be collected on the filter; in this rare situation, these circulating cells disclose anisonucleosis, irregularity of the nuclear border, large nuclei and three-dimensional sheets which are very difficult to differentiate from true CTC (20). Naevic benign cells may also be present on the filter: De Giorgi *et al*. using ISET® filtration device reported the case of a patient with a congenital melanocytic benign nevus presenting binucleated and multinucleated circulating cells with malign criteria (21). In our experience (personal data) we detected some clusters of atypical cells using Screencell® device in patients with pancreatitis. Same detection of non-malignant circulating cells has been reported in benign pancreatic tumor (22).

Applications

Immunophenotyping

Immunocytochemical and immunofluorescent techniques can be performed on the filter in order to substantiate CTC nature or to characterize it. Many studies have used immunophenotyping to confirm the epithelial (23-25), melanocytic (26), hepatic (17), adrenocortical (27) or mesenchymal (28) nature of the suspected circulating cells. In our experience, CDX2 staining can be used to indicate CTC colorectal origin (*Figure 2*) (29). Most teams studied the epithelial-mesenchymal transition in the CTC using double immunostaining with a mesenchymal (vimentin, neural cadherin) and epithelial marker (16,18,30-32). Using this procedure, Hou *et al*. have shown that microemboli have a mesenchymal phenotype and have fewer images of

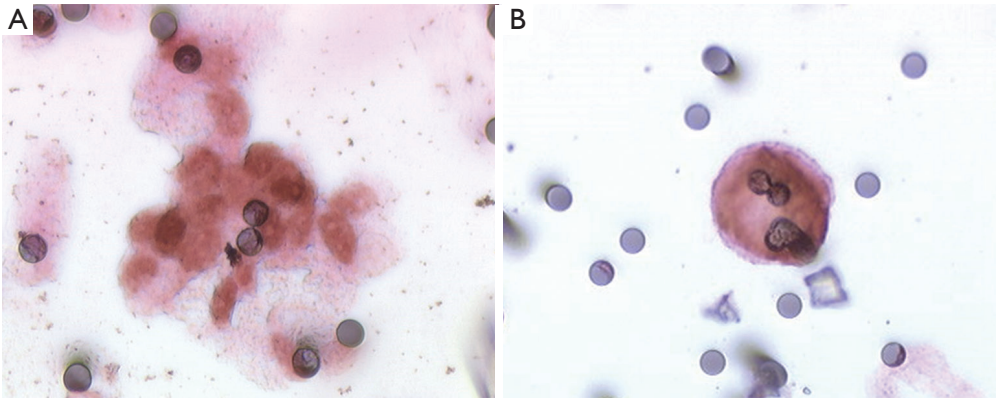

Figure 2 Anti-CDX2 nuclear immunostaining: (A) HCT116 cell line (B) circulating tumor cells (CTCs) in a patient with colorectal cancer (Obj. × 40).

apoptosis than isolated cells (31). Paul Hofman's group performed BRAFV600E and ALK immunostaining in melanoma and lung adenocarcinoma respectively allowing identification of these theragnostic somatic mutations in CTC (33,34). Abdallah *et al*. performed thymidylate synthase immunostaining on CTC in patients with metastatic colorectal cancer; this enzyme is associated with 5-fluorouracile resistance (35). Numerous studies have confirmed the variability of immunophenotype among CTC confirming their heterogeneity (16,26,31,32). Using Ki67, a marker of all active phases of the cell cycle, it has been demonstrated that microemboli are non-proliferative unlike isolated cells (13). Detection of 5-aminolevulinic acid in CTC indicate presence of living CTC which are potentially responsible for metastasis (36).

Culture

Specific devices are used for culture because of the necessity of collecting viable cells. Screencell® has developed filters with a lower pore size (6.5 µm) combined with an alternative dilution buffer for procedures not requiring fixation. The filter is then filed directly in a culture plate with classical culture medium. Cells were grown on the bottom of the well and on the filter. Recently, Metacell® has developed a size-based separation device driven by capillary-action (8). The filter is a polycarbonate membrane with 8 µm pores. Immediately after filtration, CTC are observed by light and fluorescent microscopy using unspecific fluorescent nuclear staining. Then, the filter maintained in a plastic ring is transferred to a culture plate.

Bobek *et al*. applied this technique in prostate (23), esophageal (24), mesothelioma (8), urothelial (37,38) and pancreas cancers (25) before performing immunostaining. Culture allows selection of viable CTCs and thus more tumor material in order to multiply downstream applications. However, culture could promote CTC dedifferentiation by selection of more viable clones. Screencell® also proposes a device for this application, but, to our knowledge, no results have been published yet.

Fluorescence in situ hybridization (FISH)

FISH can be performed on the filter with an adapted method. This method was applied in lung adenocarcinoma by the P. Hofman team in association with immunofluorescence in order to explore *ALK* rearrangement in CTC (34). Paillier *et al*. demonstrated that *ALK*-rearranged CTC harbored a mesenchymal phenotype and could represent a sub-cellular clone arising from a heterogeneous tumor (39). Awe *et al*. performed FISH on CTC with a telomeric probe: using a three-dimensional image acquisition system allowing the study of 3D telomeric organization reflecting chromosomic instability, they were able to assess degree of malignancy. Thus subpopulations of CTCs can be classified according to their 3D telomeric properties (40). Similarly, we performed FISH analysis in pancreatic cell line in our institution, in order to demonstrate polysomy (*Figure 3*). Indeed, in such settings, FISH can confirm the tumoral nature of CTCs in patients with pancreatic tumor.

DNA analysis

Screencell®BM specific device has been commercialized

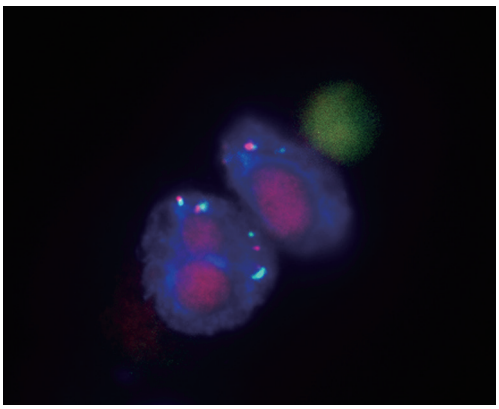

Figure 3 Fluorescence *in situ* hybridization on PANC1 cell line with DNA Vysis EGFR (epidermal growth factor receptor)/CEP 7® (centromere enumerating probe) (Abbott®) (Obj. ×40): four spots of each color are present, demonstrating chromosome seven polysomy.

to perform molecular biology. In this device, the filtration buffer is very similar to that of the culture device. The filter is a capsule adaptable on a collection tube. After filtration, lysis buffer is directly inser in the capsule-filter and products of lysis are then collecting in the tube by centrifugation. Then, classical steps of DNA or RNA extraction are performed.

However, if the number of the collected CTC is low, microdissection could be necessary to improve sensibility. In liver cancer, Vona *et al*. isolated CTCs from filter using laser microdissection capture: *β-catenin* gene mutations were analyzed by nested PCR followed by Sanger sequencing (41). Mutations were detected in 3 of 10 patients and only in a unique microdissected CTC for each patient emphasizing either technical bias or tumoral heterogeneity. Pinzani *et al*. measured *HER-2* amplification by real-time PCR on breast cancer laser microdissected CTCs (14): 4 of the 7 patients presented *HER-2* amplification in CTCs and in their matched primary tumors. Xu *et al*. employed a microfluidic platform using a slot pore microfilter to detect telomerase activity by real-time PCR-based telomeric repeat amplification protocol from filtered CTCs collected from metastatic prostate cancer patients (42).

But since demonstration of the feasibility of molecular biology techniques on CTCs isolated by filtration methods in 2000 (7), surprisingly, only few studies have shown results in patients. These difficulties are confirmed in our experience: in patients who presented KRAS mutated colon or pancreatic cancer, we failed to find exon 2 KRAS mutations in CTCs using SNaPshot® and QuantStudio® 3D Digital PCR System by Life Technologies® with or without microdissection. In these cases, we were only able to detect wild type *KRAS* sequences. One explanation for this lack of results could be the difficulties in performing these molecular techniques: they require a great number of steps on a very small number of CTCs. In the near future, this bias could be reduced using microfluidic devices. An alternative solution could be studying free circulating DNA: it appears that tumor circulating DNA allows easier and more reliable genomic analysis using new sensitive techniques such as digital PCR or next generation sequencing (43).

RNA analysis

Chen *et al*. using Screencell® filters studied epithelial-mesenchymal transition-related genes in CTCs arising in patient with prostate cancer (44): after complex micromanipulations in single isolated cell, they showed that genes promoting mesenchymal transition like *IGF1*, *IGF2*, *EGFR*, *FOXP3*, and *TGFB3*, were commonly expressed in CTCs, whereas epithelial genes were not.

Clinical interest

CTC as a prognostic biomarker

In two studies using filtration devices in adrenocortical and pancreatic cancers, CTC prognosis value was not reached; the low number of patients could explain this lack of conclusive prognostic value (16,45). However, Hofman *et al*. in their study on 204 patients with lung cancer, showed that the presence of CTCs detected by ISET® was an independent prognostic factor for shorter disease free survival (HR 1.372; 95% CI, 1.123-3.286%; P=0.006) (18). This team demonstrated that the presence of more than 50 CTCs was associated with shorter overall- and disease-free-survival (P=0.002; P=0.001) regardless of disease staging (46). In 31 patients with uveal melanoma, Mazzini *et al*. found a significant difference between overall- and disease-free-survival in stratifying groups of patients with more than 10 CTCs and with or without microemboli in univariate analysis (P=0.012; P=0.017) (47). In a series of 44 patients with hepatocellular carcinoma, Vona *et al*. demonstrated that presence (P=0.01) and number of CTCs and microemboli (P=0.02) correlated significantly with shorter survival (41). In a cohort of 131 patients with breast

cancer, Wong *et al*. showed that in early breast cancer, there was an association between CTC level (CTC > or =4) and time to progression without statistical significance (P=0.05); in metastatic disease, median CTC ≥13 independently predicted time to progression (P=0.02) but no relationship between CTC level and overall survival was found in this subgroup (48).

Taken together, these studies emphasize that CTC isolation by filtration could be a good prognostic marker for several cancer localizations, but more studies with a greater number of cases are mandatory to consolidate this promising prognostic maker.

CTC in clinical follow up

Chinen *et al*. compared ISET® and a cytokeratin (CK)-dependent immunomagnetic separation method (Miltenyi®) in the follow-up of a patient with an undifferentiated lung cancer (32). Increase in CTC number correlated with worsening clinical status of the patient, while immunomagnetic technique showed a decrease in detected cells (maybe due to EMT phenomena). The authors suggested that filtration technique was superior to immunomagnetic separation in this clinical setting.

CTC as a predictive biomarker

Chen *et al*. in their study of CTC gene expression, showed that a subset of *EMT-related* gene was more expressed in castration-resistant prostatic cancers than in castration-sensitive cancers (44). So, a molecular signature could be interesting to predict castration sensitivity and thus optimize choice of therapeutic strategies. For theragnostic purposes, analysis of *ALK* translocation in lung cancer (34,39), *BRAF* mutation in melanoma (33) and *HER-2* amplification in breast cancer (14) is feasible in CTCs. Thymidylate synthase CTC immunostaining is associated with metastatic colorectal cancer progression after 5-Fluorouracil treatment and therefore could predict resistance to this treatment (35).

Predictive makers can be detected using immunocytochemistry, therefore it is likely that these procedures are transposable to CTCs in a patient care setting. These approaches are promising because they could provide a more accessible source of tumor tissue compared to primitive or metastasis tumor tissues, allowing real-time monitoring of patient mutation status. Also CTC could be more representative of cancer heterogeneity than a fragment of tumor. But more studies with a greater number of cases are necessary to evaluate sensitivity, efficiency and real clinical benefit of these techniques.

CTC in diagnosis

CTC isolated by filtration could be of interest for the diagnosis of tumors which are difficult to sample. Our group has evaluated the diagnosis benefit of CTCs in pancreatic tumor (49). Sensitivity and specificity were 55.5% (95% CI, 40.1-70.9%) and 100% (95% CI, 75-100%), respectively. With a diagnosis accuracy of 70%, CTCs provide a noninvasive alternative to fine needle aspiration in pancreatic tumor. Hofman *et al*. studied CTC in 250 patients undergoing surgery for non-small cell lung carcinoma (50): 102 patients presented CTCs with malignant criteria before surgery with a low inter-observer variability. In another study on 76 broncho-pulmonary cancer patients, Freidin *et al*. found a 71.9.0% (95% CI, 60.5-83.0%) sensitivity and a 52.9% (95% CI, 31.1-77.0%) specificity of CTCs (51). A cohort of 77 patients with malignant and benign lung diseases was studied by Fiorelli *et al*. who showed that for a malignant circulating cell count greater than 25, sensitivity and specificity were 89% and 100%, respectively (52). In 72% of CTC positive patients, immunohistochemical analysis provides a specific histologic diagnosis of the corresponding primary tumor. Pinzani *et al*. provided the evidence that CTCs may represent a marker to support differential diagnosis between adrenocortical carcinoma and adrenocortical adenoma (27,45).

CTC detection could show an interest in cancer screening: indeed, there can be several months to years between initiation and detection of a tumor. CTCs can be present at the beginning of tumor development (22,53-55). Ilie *et al*. searched for CTCs in patients with chronic obstructive pulmonary disease which is a predisposing factor for lung cancer (56): five patients presented CTCs without any clinical or radiological proof of tumor and were annually followed by low-dose spiral computed tomography. For all these patients, lung nodules were detected 1 to 4 years after CTC detection, corresponding to early-stage lung cancer at histology. These sentinel CTCs could predict the occurrence of cancer in high-risk populations and could be used in tumor screening programs.

Perspectives: microfluidic devices

Size-selective microfluidic systems enable more precise and more efficient CTC enrichment than traditional filtration

devices (57). Indeed, these nanotechnology structures, handling microliter amounts of liquid circulating in channels less than a tenth of a micrometer, allow more efficient CTC filtration (58-60). Furthermore microfluidic devices can facilitate downstream exploitation of CTCs (42,58,61). However, these new promising systems must be tested on large series of cases in order to validate their performance.

Conclusions

Filtration methods are sensitive and easy techniques which allow cytomorphological and immunocytochemical analysis of CTCs. However, due to the absence of multicentric studies, CTC clinical applications are not robust. Biological exploitations seem easy but are still difficult to develop requiring adaptation of known techniques for these very specific cells. Alternative free circulating DNA seems to be more accessible for molecular biology characterization. Developments of novel microfluidic techniques are promising and will, perhaps, allow greater clinical perspective.

Acknowledgements

The authors are grateful to Nikki Sabourin-Gibbs, Rouen University Hospital, for her help in editing the manuscript.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Dolfus C, Piton N, Toure E, Sabourin JC. Circulating tumor cell isolation: the assets of filtration methods with polycarbonate track-etched filters. Chin J Cancer Res 2015;27(5):479-487. doi: 10.3978/j.issn.1000-9604.2015.09.01

