Am J Cancer Res 2021;11(9):4111-4126
www.ajcr.us /ISSN:2156-6976/ajcr0136241

Review Article

Current methodologies to detect circulating tumor cells: a focus on ovarian cancer

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Received June 10, 2021; Accepted July 19, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Identification of circulating tumor cells (CTC) in liquid biopsies opens a window of opportunities for the optimization of clinical management of oncologic patients. In ovarian cancer (OC), which involves atypical routes of metastatic spread, CTC analyses may also offer novel insights about the mechanisms behind malignant progression of the disease. However, current methodologies struggle to precisely define CTC number in the peripheral blood of OC patients, and the isolation of viable cells for further characterization is still challenging. The biggest limitation is the lack of methodological standardization for OC CTC detection, preventing comprehensive definition of their clinical potential required for the transfer to practice. Here we describe and compare methods for CTC analysis that have been implemented for OC thus far, discussing pros, cons and improvements needed. We identify biophysical separation approaches as optimal for CTC enrichment. On the other hand, the identification of specific tumor antigens or gene transcripts, despite displaying drawbacks related to tumor heterogeneity, still remains the best approach for OC CTC detection.

Keywords: Circulating tumor cells, ovarian cancer, methods, liquid biopsy

Liquid biopsy for circulating tumor cell analysis in ovarian cancer

Obtaining diagnostic and prognostic information by simply performing a blood test has long been the holy grail in oncology. Today, liquid biopsy is used for clinical evaluation in several cancer contexts, mainly by detecting circulating tumor-derived cell free material (DNA, RNA, proteins, metabolites, exosomes) [1]. In addition, peripheral blood may also contain circulating tumor cells (CTCs) that are shed from the primary tumor, but escape anoikis, i.e. death of anchorage-dependent cells when detached from the tissue of origin. These rare entities (<10 per mL) survive in circulation for long periods, eventually nesting in distant tissues and forming metastases. Having a longer life span than cell-free material, CTCs may provide additional clinical benefit, aiding patient follow-up. Moreover, CTC detection may be used for a non-invasive early diagnosis, which would be particularly ground-braking in the context of ovarian cancer (OC), where screening protocols are still lacking and 70% of patients is diagnosed at the advanced stages of the disease, resulting in high mortality [2]. Finally, it is clear that the information held in CTCs is of great value to understand biological mechanisms of metastasizing, which in OC involves atypical routes still to be elucidated. Nevertheless, the actual clinical significance of CTC analysis has been somewhat contested in OC, since the metastatic spread of this malignancy is considered to occur predominantly locally, via direct transcoelomic dissemination to the omentum, while the peripheral hematogenous route, which would involve the presence of CTCs, is less common [3]. Thus, it was initially assumed OC is not the optimal context for the use of CTCs for diagnostic/prognostic purposes. The emerging evidence reports primary tumor shedding to peripheral blood even in patients diagnosed with early-stage OC [4, 5], and shows that development of omental metastases may actually be the result of both transcoelomic and hema-

togenous spread [6-8]. Concordantly, the last decade has witnessed a vast number of works reporting relatively high rates of CTC-positive OC patients (60-98%), implying correlations may be drawn with various clinical parameters, such as monitoring disease and drug resistance [9-13]. In addition, several meta-analyses have been conducted, mainly concluding that the presence of OC CTCs correlates with worse overall and progression-free survival [14, 15]. However, the comprehensive definition of OC CTC clinical potential required for the transfer to practice is still lacking, mainly due to the scarce methodological standardization for their detection.

Here we discuss methods for CTC analysis that have up to date been implemented in OC, distinguishing enrichment from detection approaches, to recognize pros and cons of available protocols and understand which directions to follow for future technological optimizations. Among other, we compare capture rate efficiencies estimated with golden standard samples generated by spiking known cancer cell numbers into healthy donor blood (**Table 1**).

Starting point: sample enrichment is the most efficient when exploiting circulating tumor cell biophysical properties

Identification of CTCs in the patient's peripheral blood is technically challenging on one hand due to their low absolute numbers and on the other because of blood cells abundance. At late cancer stages, CTCs were reported at frequencies of 1-10 per mL of whole blood [5, 16], while the same volume contains also 5×10^9 of erythrocytes and 10^7 of white blood cells. Thus, the majority of investigations require an enrichment step to increase CTC concentration before detection. Separation can be based on distinct biophysical properties between blood cells and CTCs, as the latter display aberrant cytological features including low deformability, altered density, larger cellular size and heterogenous nuclear shape. Alternatively, antibodies can be used to either label immune cells for depletion or bind cancer specific markers for positive selection. In this chapter we discuss pros and cons of various enrichment procedures used in the context of OC. We recognize methods based on biophysical properties as the currently most promising approach, especially when samples are processed on microfluidic devices, since they allow recovery of heterogeneous populations of intact CTCs, which is crucial for confident functional characterization and molecular profiling.

Microfiltration

Based on differences in morphology and size between cancer cells (15-40 μm in diameter) and leukocytes (≤10 μm in diameter), microfiltration offers a simple and rapid enrichment, by processing large blood volumes within minutes, with minimal pre-handling. Of note, CTC detection is often possible directly on the filtration membrane. Several small-format microfiltration devices have been developed, some of them applied for OC CTCs recovery. In particular, one group used the CanPatrol system, based on vacuum filtration through a membrane with 8 μm diameter pores [17]. When spiking 10-200 cancer cells in 5 mL of blood, CanPatrol has been reported to provide recovery rates ranging from 80 to 89% [18] (**Table 1**). However, the approach does not allow retrieval of vital CTCs, since they are formaldehyde-fixed before filtering. Alternatively, obtaining viable CTCs is possible when using the small bench instrument MetaCell™ whose absorbent polycarbonate membrane allows capillary-action driven passage of the plasma, whereas the blood cells are filtered through the 8 μm diameter pores. The CTC fraction is observable immediately after isolation and can be cultured *in vitro* for further enrichment [19, 20], or subjected to cytomorphological evaluation and gene expression analysis for molecular characterization [19-21]. To the best of our knowledge, there is no literature reporting evaluation of MetaCell™ recovery rates. A similar technology called ISET (Isolation-by-Size-of-Epithelial-Tumor), which also captures CTCs on a polycarbonate 8 μm pore membrane, was shown to present with high recovery rates (83-99%) when 1-100 breast cancer cells were spiked per mL of blood [22] (**Table 1**). Even though ISET seems to be frequently used for CTC enrichment in other tumor types [23-25], to the best of our knowledge only one published study applied the technology in OC [26].

Another filtration approach, used by Kim and colleagues for the CTC isolation from OC patients, is the tapered-slit filter (TSF) which contains either 6 or 8 μm pores, wide at the entrance and narrowing with depth to minimize

Table 1. Recovery rates of spiked cancer cells captured by different enrichment/detection methods. When available, studies using ovarian cancer cell lines were preferably cited

Method	cells/mL blood	Recovery rate	Tumor model	Reference
CanPatrol	2-40	80 to 89%	HepG2 cell line (hepatocellular carcinoma)	[50]
ISET	1-100	83 to 99%	MCF7, SKBR3, and MDA-MB-231 cell lines (breast cancer)	[22]
Tapered-slit membrane filter (TSF)	2000	82%	H358 cell line (lung carcer)	[27]
OncoQuick™	2.7×10^5	87%	HT-29 cell line (colorectal carcinoma)	[35]
	5000	21%	KB cell line (nasopharyngeal cancer)	[16]
RosetteSep™	5000	62.5%	KB cell line	[16]
	2-50	40%	MDA-MB-231 cell line	[36]
Negative leukocyte depletion using CD45 magnetic beads	40	32% to 77%	CAL-54 and A-498 cell lines (renal and kidney carcinoma, respectively)	[38]
Automatic CD45 depletion	100	40% to 82%	BG-1 cell line (ovarian cancer)	[39]
3D-printed microfluidic device for CD45 depletion	3×10^3-4×10^3	90%	HeyA (ovarian cancer), LNCaP (prostate cancer) and MDA-MB-231 cell lines	[40]
CD-PRIME™	13-50	85-87.5%	SKOV3 and OVCAR3 cell lines (ovarian cancer)	[48]
Parsortix™	5	28-72%	TOV21G and CaOV3 cell lines (ovarian cancer)	[84]
	7	69%	MDA-MB-468 cell line (breast cancer)	[53]
3D-printed microfluidic chip	<10	56%	SKOV3ip1 (ovarian cancer)	[54]
IsoFlux	3-40	57-83.5%	SKOV3 cell line	[45]
CellSearch®	1	80-82%	SKBR3 cell line (breast cancer)	[71]
	1-67	42-75%	SKBR3 (high EpCAM) and CAL-120 (low EpCAM) cell lines (breast cancer)	[46]
Biotin-doped Ppy-deposited Microfluidic Device	10-100	>65%	HCT116 (EpCAM-positive) and T24 (EpCAM-negative) cell lines (colon and bladder cancers, respectively)	[79]
AdnaTest Ovarian Cancer Detect	1-5	100%	OvCar3 cell line (ovarian cancer)	[62]
Microfluidic glass chip	50, 100 and 1000 cells in 500 µl	88%	OvCar3 cell line	[90]

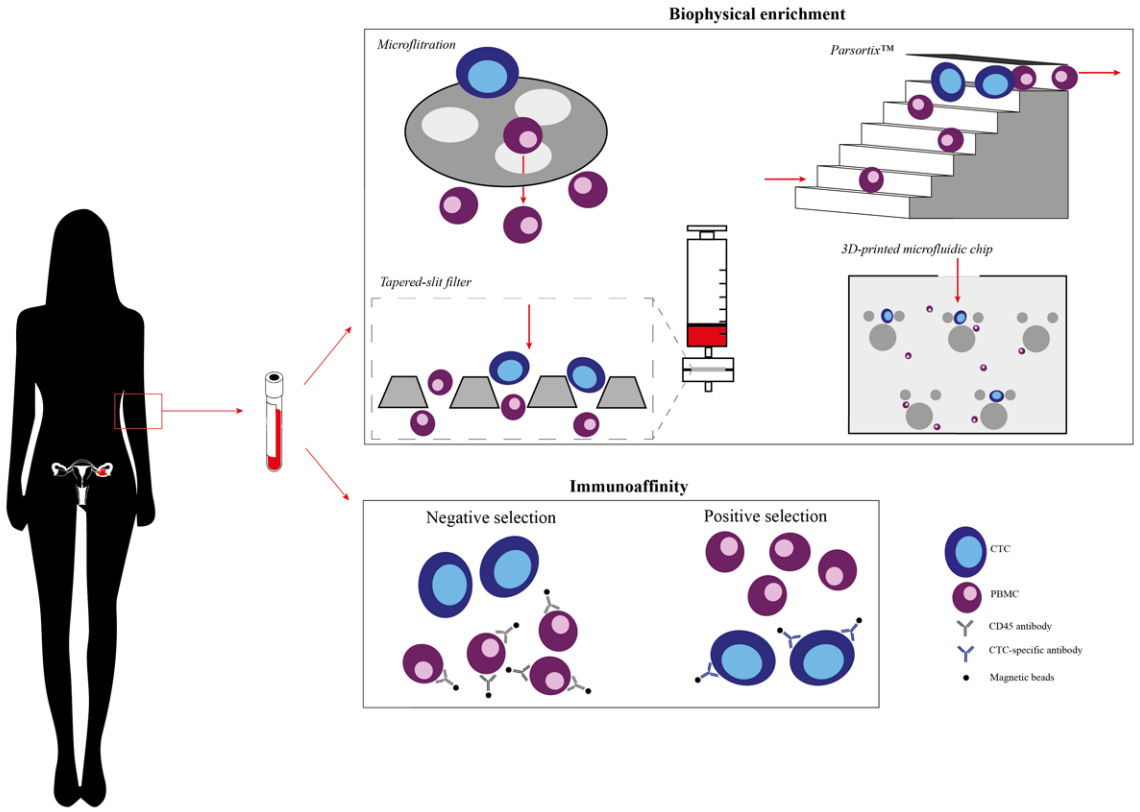

Figure 1. An overview of CTC enrichment approaches. In the upper panel, microfilters and microfluidic chips are represented which discriminate CTCs based on their biophysical properties by using various types of "traps" to reduce cell stress and clogging during flow-through: tapered-slit filter system consists of conical pores, Parsortix™ cassette uses wide separation walls, while Guo and colleagues [54] developed a chip with three-column traps. Red arrows indicate the direction of the blood sample flow-through. Immunoaffinity methods are represented in the lower panel, which include either the negative CTC selection via depletion of CD45-positive leukocytes, or the positive immunocapture by binding CTC-specific antigens, such as EpCAM or mucins.

cell stress [13, 27] (**Figure 1**). When using 6 µm filter and by spiking 2000 cells/mL of blood diluted with PBS (1:4), 82% recovery rate was obtained (**Table 1**) [27].

In theory, microfiltration is a simple and rapid enrichment approach, without requirement for sample preparation steps, allowing the capture of both single CTCs and oligocellular clusters with relatively high recovery rates. However, it must be noted the healthy donor's blood used in spiking experiments harbors lower immune cell numbers than what is found in cancer patients, whose samples more easily cause clogging in filtration systems. Moreover, the shear pressure of the flowing cell suspension can damage trapped cells, reducing CTC viability [28], which is sometimes furthermore compromised by difficult detachment from the filtration membrane. Finally, when staining is performed directly on the filter, the background signal may limit subsequent detection.

Density gradient centrifugation

The high nucleus to cytoplasm ratio results in a specific sedimentation coefficient of cancer cells, allowing their enrichment from whole blood by density gradient centrifugation. In particular, CTCs and monocytes (density <1.077 g/mL) are separated from other blood cells (density >1.077 g/mL) [29]. It is a fast and inexpensive procedure, which is why it has initially been used widely for CTC enrichment, also in studies analyzing OC patients [9, 30, 31]. However, samples obtained by density gradient centrifugation result in high leukocyte contamination [32]. To increase cell purity, systems such as OncoQuick™ were designed, where a porous barrier is positioned above the density gradient for additional separation by filtration [33, 34].

Even though a high recovery rate (87%) has been initially reported with such system, it must be noted the study used a disproportionally high number of spiked cancer cells (>2 × 10^6 in 10 mL) [35] (**Table 1**). Indeed, when the spiking numbers are lowered, the resulting recovery is much less efficient (21%) [16]. Alternatively, immunolabelling of blood cells with the RosetteSep™ kit may be performed to crosslink leukocytes and erythrocytes, altering their sedimentation coefficient for more efficient CTCs separation during density gradient centrifugation. He and colleagues compared nine different density gradient procedures (Ficoll-Plaque, A23187 treatment plus Ficoll-Plaque, RosetteSep™-Ficoll, Ammonium chloride lysis, Histopaque 1077, 1083 or 1119, OncoQuick and LeucoSep tube with Ficoll), and found that applying RosetteSep™ procedure yields the highest OC CTC recovery efficiency (62.5%) [16]. However, these data are to be considered with caution since the authors used high cancer cell concentrations (5000 cells/mL) for the evaluation of enrichment efficiency. Studies using more appropriate numbers, similar to the realistic conditions found in cancer patients (2-50 cells/mL), report RosetteSep™-Ficoll recovery rates of 40% [36].

Taken together, density gradient approaches currently remain relatively inefficient for enrichment of samples containing low CTC numbers (<10/mL), as cells are lost during the procedure, either due to the cytotoxicity of the medium, or due to the aggregate formation altering the sedimentation coefficient. Consequently, CTC clusters recovery is also not effective by density gradient separation.

Negative immunoaffinity for CD45 antibody

Negative immunoaffinity enrichment methods imply the depletion of unwanted immune cells from the blood by targeting leukocyte-specific antigen CD45 that is not expressed by the CTCs (**Figure 1**). The approach is usually preceded by either red blood cell lysis or gradient separation to remove erythrocytes. Next, the sample is incubated with anti-CD45 tethered to magnetic particles and exposed to a magnet which retains the labelled leukocytes. CTCs are found in the eluate and may be further purified for downstream applications such as RT-qPCR assays [37]. Recovery rates reported for negative leukocyte depletion using CD45 magnetic beads show considerable variability, ranging from 32% to 77% depending on the cell type [38] (**Table 1**). Automated versions of this approach have been developed to allow one step enrichment. For example, Tsai and colleagues validated an integrated microfluidic system capable of simultaneous red blood cells lysis, CTC enrichment via CD45 leukocyte depletion, and even subsequent detection of OC CTC [39]. The average recovery rates reported for this system range from 40% to 82%, for samples with 100 cancer cells per mL [39] (**Table 1**). Apart from the most commonly applied magnetic separation, custom-made platforms have been developed where CD45 antibodies are immobilized to the device surface, retaining the leukocytes as the sample passes through. In particular, Chu et al developed a 3D-printed microfluidic system functionalized with the CD45 antibody to deplete white blood cells and integrated with a 3 μm micropore filter downstream of the immunodepletion to capture CTCs [40]. The device has been tested with spiked samples, including OC cells, with promising results reporting 90% recovery rate [40] (**Table 1**). However, recovery of low CTCs numbers (< 3000/mL) has not been tested.

The main advantage of the CD45-based negative selection methods is that they are not biased for specific CTC populations, allowing enrichment of heterogeneous CTC types, irrespective of their size or density. On the other hand, purity of CD45 negative enrichment is relatively low, because even though the depletion rates reach 99%, this still leaves up to three million unwanted cells, especially in cancer patient samples that harbor particularly high leukocyte numbers [41]. Moreover, CD45 negative selection may reduce recovery, since CTC clusters containing immune cells risk to be depleted [42]. Lastly, few reports indicate that even cancer cells themselves may express the CD45 antigen [43].

Positive immunocapture

Instead of labelling immune cells for depletion, enrichment via positive immunocapture is based on targeting CTC specific markers (**Figure 1**). In OC, this is most often achieved by using antibodies against epithelial cell adhesion molecule (EpCAM) generally expressed in epithelial neoplasia. Several EpCAM enrichment systems are commercially available. For

example, CellSearch® uses ferrofluid nanoparticles functionalized with EpCAM antibody for the magnetic separation of labelled cells [44]. Moreover, Isoflux™ combines a microfluidic technology with immunomagnetic beads preconjugated with EpCAM antibody for the separation of CTCs while passing through a flow channel traversing a magnetic field [45]. These systems may be integrated with automated CTC enumeration by immunostaining additional markers, but in circumstances when other specific downstream applications are required, the elution of the CTC enriched sample is possible, with reported recovery rates varying between 40-75% if 10-500 cells were spiked in 7.5 mL of healthy donor blood [45, 46]. To increase enrichment efficiency, more than one antibody may be used for immunocapture, as is the case in AdnaTest Select system which, for OC, apart from EpCAM, labels Mucine 1 (MUC1) and Mucine 16 (MUC16). Of note, AdnaTest offers kits specialized for various cancer types and, instead of immunostaining, combines gene expression analysis for CTC detection [45, 46].

Even though the purity of positive immunocapture is usually higher than that of other enrichment methods, the most relevant limitation is the inability to capture CTCs with low or no expression of epithelial markers, which is the case in certain OC subtypes [47].

Label-free microfluidic-driven separation

In recent years, several microfluidic devices have been developed, in which a precisely controlled pressure is applied to process liquid biopsy through miniaturized analytic systems containing submillimetric traps made of chambers or tunnels (**Figure 1**). CTCs are captured based on their larger size and lower deformability, compared to the unwanted blood cells which are flushed through. Flow rate fine-tuning ensures minimal cell stress, maintaining CTC viability. Of note, similarly to microfiltration, these enrichment procedures often allow "on the spot" labelling for immediate subsequent detection and enumeration of CTCs, reducing cell loss during elution steps. For example, CD-PRIME™, a commercially available centrifugal microfluidic disc exploits fluid-assisted separation technology (FAST) to capture CTCs on a 8 μm pore polycarbonate membrane, where direct immunofluorescent staining is possible [48]. The disk is loaded with 3 mL of the sample and spun in a table-top centrifuge-like instrument (CD-OPR-1000TM), allowing rapid processing of whole blood (3 mL/min). The centrifugal force combined with the specific disk design results in tangential flow filtration and uniform pressure drop applied across the whole membrane, minimizing clogging and CTC damage. This system showed a mean capture efficiency of 84.7-87.5% when analyzing 40-150 spiked cancer cells per 3 mL of blood (**Table 1**). Contrariwise from the pore-based microfluidic separators, the Parsortix™ system uses a microscope slide-sized disposable cassette, which contains a series of stair-like walls ending with a wide channel of specific height (6.5-10 μm) (**Figure 1**), where CTCs are trapped due to their large size and low deformability [9, 49, 50]. While passing through the Parsortix™ cassette, cancer cells are submitted to a negligible flow restriction due to the width of the channel, preventing the elevated shear stress typical of pore-filtration methods. This gentle capture maintains structure and viability of CTCs, and has been shown to preserve CTC clusters [51]. After the procedure, blood cells are not completely eliminated, but their numbers are reduced by 10^6-fold [9], leaving 200-5000 unwanted leukocytes [52]. Captured CTCs may be stained in the cassette and visualized under the microscope for enumeration. Alternatively, the cells can be flushed either with lysis buffer for subsequent molecular analysis or with PBS to allow viable CTC recovery for culturing. Up to date, one study reported separation of OC CTCs from both spiked samples and OC patients liquid biopsies using Parsortix™, showing that such enrichment approach allows downstream detection of as few as 5 OC cells in mL of blood [9]. Importantly, higher recovery rates are observed when cassettes with smaller gap height are used (69% with 6.5 μm compared to 34% with 10 μm gap), indicating size heterogeneity (**Table 1**) [53]. Another microfluidic device that captures CTC based on their size and deformability has been developed by Guo and colleagues [54]. In their system, a 3D-printed microfluidic chip consists of 8 chambers, each containing about 700 capture sites of different sizes (8, 10 and 15 μm), which serve as CTC traps (**Figure 1**), while smaller red blood cells and most leukocytes pass through [55]. The device contains also a

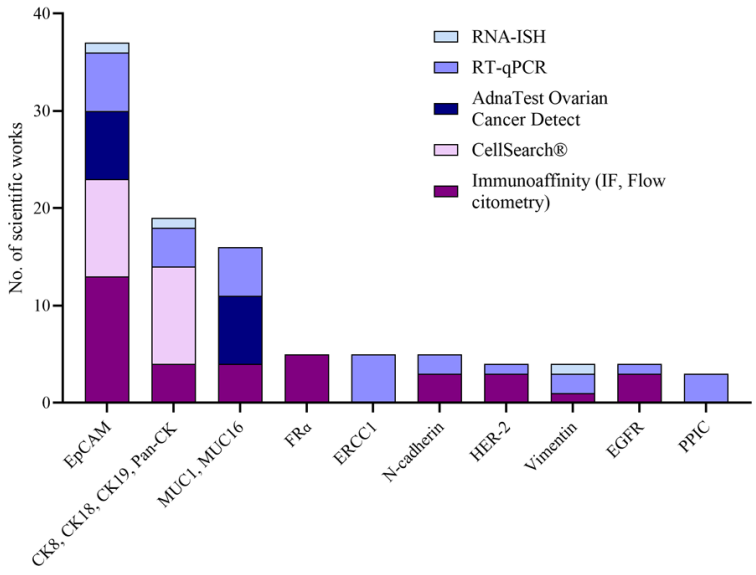

Figure 2. Molecular markers most frequently used for OC CTC detection. Methods identifying gene and antigen expression are indicated in blue and purple, respectively. The reported molecular markers have been used in at least four studies performed by different scientific groups. IF = immunofluorescence, CK = cytokeratin.

pre-filter that prevents clogging by large debris. Compared to CD-PRIME™ and Parsortix™, which allow direct blood processing, Guo and colleagues apply plasma removal and dilution of the cellular blood fraction in 1 mL of buffer solution. Their procedure of combining the microscopic traps with the application of fine-tuned flowrates was capable of detecting <10 OC cells/mL of blood, with an average capture efficiency of 55.7%.

Apart from being capable to detect heterogeneous CTCs from the molecular profile point of view, the microfluidic enrichment offers a few additional advantages. For instance, it is characterized by automation capability and high flexibility to accommodate downstream technologies. In addition, the possibility for fine-tuning the flowrate contributes to minimizing clogging and, most importantly, allows gentle separation, maintaining CTC viability and integrity. Thus, it is somewhat unexpected to find lower recovery rates for microfluidic devices compared to microfiltration (**Table 1**). It is important to note the two approaches have not been directly compared and recovery rate evaluation by spiking into healthy donor blood is generally limited due to the lower amount of white blood cells in such samples than that usually found in oncologic patients. The purity of the sample obtained by microfluidic devices is still an issue, as CTCs are retained together with a substantial portion of immune cells. While this may be resolved by immunofluorescent staining, the latter is not useful if the isolation of viable CTCs is required.

CTC detection in OC patients depends primarily on molecular marker analyses

Whereas the aforementioned enrichment approaches perform most efficiently when exploiting CTC biophysical properties, their identification and enumeration is currently achieved mainly by molecular analyses, such as detection of specific DNA mutations, protein markers or gene transcripts. In OC, as for other epithelial neoplasms, this mainly involves recognition of EpCAM, and other epithelial markers such as cytokeratin (CK) (**Figure 2**) [56, 57]. However, despite the high sensitivity of such approaches, their main drawback remains the necessity of the *a priori* knowledge about the CTC molecular profile, which is challenging due to the tumor heterogeneity. Even within the same type of cancer, the biology of cells shedding from the primary site is variable, depending on the tumor subtype, stage and grade. Indeed, CTCs have in recent years experienced an identity crisis: their initial definition as nucleated cells expressing EpCAM and lacking the leukocyte antigen CD45 is being continuously updated (**Figure 2**), as EpCAM- and CK-negative CTCs have been discovered and new data suggest high CTC heterogeneity due to the dynamic cellular reprogramming during metastasizing [58]. Indeed, both EpCAM and CK are often found downregulated as part of oncogenic adaptation to allow epithelial cell dissociation from the tumor, and facilitate cell plasticity and migration, respectively [59, 60]. This might lead to underestimation of CTC numbers when using epithelial marker-based detection, jeopardizing clinical decisions. With advancement of our knowledge on metastatic processes in OC, it is now clear that CTCs often present with mesenchymal features that increase invasiveness

and metastatic potential [61]. Moreover, recent evidence suggests that OC CTCs may display not only EMT-associated transcripts, but also stem cell markers [17, 62], and are often characterized by high folate receptor-alpha (FRα) expression [63]. Thus, studies applying simultaneous detection of several proteins have shown to increase CTC detection rate in OC patients. For example, the simultaneous detection of EpCAM and FRα significantly increased the CTC capture rate to 92%, exceeding the capture rate obtained with EpCAM or FRα targeting alone by 20% [63]. Similarly, combined targeting of EpCAM and mesenchymal marker N-cadherin increased OC CTCs detection by approximately three times compared to targeting EpCAM alone [64]. However, it must be noted that N-cadherin is also expressed on the circulating endothelial cells, potentially leading to false positive results if simultaneous staining of vascular endothelial cadherin is not performed to distinguish the two entities [64]. On the other hand, the selection of these false positives is rare with EpCAM targeting [64]. Of note, OC CTCs co-expressing epithelial and mesenchymal phenotypes have been reported to increase after chemotherapy, highlighting the dynamic protein expression profile and the need for inclusiveness of various markers to maximize CTC detection [65]. Thus, it is not surprising there are currently no known universal biomarkers, warranting confident identification of all CTCs in a liquid biopsy from OC patients. The majority of the literature analysing CTCs in OC has been based on targeting EpCAM and CKs (**Figure 2**), which might have contributed to inconsistent results when analysing the association of CTCs with progression free and overall survival [66-69]. Here we discuss the most commonly used molecular approaches for CTC detection in OC patients, and describe how the spectrum of the recommended markers is evolving, as the technical progress allows more insight on CTC biology, opening the window of eventually developing label-free CTC detection.

Epitope recognition-based methods

One of the most widely used approaches for CTC detection is the labelling of specific tumor-associated antigens. This has mainly been done by fluorescent immunocytochemistry, often directly on the membrane/chip used for enrichment [19, 21, 34, 45, 48, 54]. Together with DAPI to identify nucleated cells, the sample is stained against CD45 and CTC-specific antigens such as EpCAM, MUC1 or MUC16 [65]. For example, Isoflux™ enrichment step has been integrated with pan-CK and CD45 fluorescent staining to identify the recovered CTCs, which are then enumerated through the automatic microscope scanning [45]. The system efficiently captures OC CTC from whole blood, also when low spiking numbers are used (average capture rate of 70% of 20-300 tumor cells spiked in 7.5 mL blood; **Table 1**). Immunofluorescence is highly specific and allows CTC enumeration, but requires substantial operator workload, which is why automated systems have been developed. For example, microfluidics-based enrichment instrument Parsortix™ has been integrated with automatic in-cassette staining protocol in which the operator may select which antibodies to use. However, the subsequent CTC count is performed manually. On the other hand, CellSearch® allows not only enrichment, but also automated CTC enumeration via in-device immunocytochemistry. In detail, after positive immunocapture with ferrofluid nanoparticles functionalized with EpCAM antibodies, cells are permeabilized and stained against CK8, CK18 and CK19, while CD45 antibody is used to exclude leukocytes and DAPI to identify nucleated cells. Next, the cells are pulled by a magnetic cartridge to a single focal depth for scanning. The approach was compared to Parsortix™ in a few studies, showing no substantial differences between recovery rates when analysing spiked samples [70], but when the two methods were used to detect CTCs in patient liquid biopsies the results were discordant with positive patients being identified exclusively with Parsortix™ or CellSearch® in 15% and 19% of the cases, respectively [52]. CellSearch® is the most well-known approach for CTC analysis and, so far, the only U.S. Food and Drug Administration (FDA) approved method for detection and enumeration of CTCs in the peripheral blood of breast, prostate and colorectal cancer patients [71]. The FDA accreditation has led to diffusion of CellSearch® as the golden standard within the CTC field, thus most of the emerging technologies validate their performance by comparison with CellSearch®. The system has been broadly used to enumerate epithelial CTCs, being employed in about 15% of the scientific works on ovarian malignancies (**Figure**

2). Moreover, it is the only approach applied for CTCs analysis in OC clinical trials [72-74]. Capture rates are typically high for EpCAM expressing cells, ranging between 80 to 82%, also when low cell numbers are added to the whole blood sample (5-10 cells/7.5 mL blood) [71]. However, it should be noted that a spiking experiment with an OC cell line is still lacking, which would be important since the capture efficiency of the CellSearch® approach strongly depends on the expression of markers used for cell recognition. Indeed, it was demonstrated that spiking the same concentrations (10-500 cells/7.5 mL blood) of high versus low EpCAM-expressing cells results in considerably different recovery rates (75% versus 42%, respectively) [46] (**Table 1**). Moreover, a study comparing ISET technology and CellSearch® for CTC detection in patients with breast, prostate and lung carcinomas revealed discrepancies between the CTC numbers isolated with the two approaches [23]. In breast cancer patients, CTC counts were generally higher by CellSearch® [23]. On the contrary, in 80% of lung cancer patients the number of CTCs identified was higher with ISET technology, which was also more efficient to detect prostate cancer CTCs, highlighting the limits of marker-specific detection. While its biased approach towards the epithelial phenotype risks underestimating the CTC count due to exclusion of cells carrying exclusively mesenchymal or stem markers, the most important advantage of CellSearch® is the possibility of simultaneous enrichment and automated CTC enumeration, which ensures standardization and reproducibility of the analyses. Of note, the same manufacturer developed the DEPArray™ platform (Di-Electro-Phoretic Array system), which after the initial staining via CellSearch®, applies dielectrophoretic forces to a mixed-cell population sample, to isolate single viable CTCs by means of a chip consisting of various microelectrodes generating electric cages in which individual CTCs are trapped. The approach allows the recovery of a single CTC available for further investigations, including molecular analyses and culturing, but is yet to be used in the context of OC [75, 76].

Besides immunocytochemistry, epitope recognition may involve also flow cytometry approaches. In particular, ImageStream platform was used to detect CTCs in stage III OC patients by staining against CK, WT1 and CD45 [77]. The advantage of the system is the possibility of staining with markers chosen by the operator. Moreover, conversely from traditional flow cytometry systems, it allows visualization of labelled cells. It should be noted, however, that the authors do not provide detection rate evaluation by spiking experiments [77]. Thus, even though the approach seems promising, the data are to be interpreted with caution, since flow cytometry traditionally lacks the sensitivity to detect underrepresented populations such as CTCs. In this context, boosting flow cytometry efficiency in detecting low OC CTC numbers has been attempted by generating antibody-bound silica nanoparticles with enhanced fluorescent ability [78]. However, the approach is currently in a developmental phase, being tested only by spiking 100 OC cells in 50 µl of blood [78].

Furthermore, a noteworthy epitope detection platform has been developed by Jeon et al, who built a nanoroughened microfluidic device in which OC CTCs were isolated via an electrically conductive chip coated with biotin-doped polypyrrole and streptavidin [12, 79]. The latter layering allows a versatile choice of various biotinylated antibodies that can be used simultaneously in one step analysis. In particular, by labelling EpCAM, TROP-2, EGFR, vimentin and N-cadherin, the authors report capture rate >65% for samples spiked with 10 cancer cells per mL of blood [79] (**Table 1**). Capture rates were at 8% when only EpCAM antibody was used for labelling, highlighting the need for multiple-marker staining. Importantly, the system allows CTC cluster detection and viable cell recovery, but has not yet been widely used.

Besides the typical use of fluorescent immunostaining, it is interesting to note that antigen-based CTC recognition has also been coupled with a micro-nuclear magnetic resonance (µNMR) technology that should in principle allow more specific detection of extremely rare events. Indeed, a decade ago Ghazani and colleagues reported µNMR detection of nanoparticles functionalized with antibodies against EpCAM, HER-2, EGFR and MUC-1, which successfully capture OC CTCs [80]. Notably, this approach showed considerably higher detection sensitivities with respect to CellSearch® and allowed one-step analysis, directly from the whole blood, without the need for sample

enrichment or cell isolation. Similarly, functionalized magnetic nanoparticles have been analysed by a microfluidic chip-based micro-Hall detector for simultaneous detection of EpCAM, HER2/neu and EGFR on individual OC CTCs [81]. Nonetheless, up to date, such analyses have not been widely used, at least not for CTCs, most likely due to requirements for fine-tuning the technology towards a user-friendly approach [82].

In conclusion, being based on antigen-antibody interaction, positive immunocapture approaches are typically highly specific. The main pitfall remains that antigen-based detection still fails to account for CTC heterogeneity. Moreover, the approach often requires cell fixation which precludes isolation of viable cells.

OC CTC specific gene expression analysis

While immunocapture permits precise enumeration, usually only a limited number of markers can be used for staining (<5), risking false negative results due to CTC heterogeneity. In this context, quantitative Real Time PCR (qRT-PCR) is a highly sensitive technique by which analysis of dozens of markers may be performed simultaneously, detecting even rare transcripts. Indeed, the detection of epithelial or tumor specific gene expression has been shown informative to detect CTCs in OC patients in various studies (**Figure 2**). In some cases, the sensitivity has been increased by introducing gene of interest pre-amplification steps and by optimization of various enrichment approaches to identify the most efficient workflow [9]. The same study obtained the most efficient enrichment results by combining density gradient with Parsortix™ separation. Moreover, qRT-PCR is the detection method of choice in the QIAGEN AdnaTest Detect kit, where *EPCAM*, *MUC1*, and *MUC16* transcripts are analysed after the initial positive immunoenrichment by AdnaTest Select [63, 83]. Samples are defined as CTC-positive if the measured quantity of at least one of the tumor markers is above a defined threshold (>0.1 ng/μL). The AdnaTest Ovarian Cancer Detect approach was demonstrated to recognize low numbers of OC cells (5-25 cells/5 mL blood) with high recovery rates (100%) [62]. This approach is so far the second most commonly used to detect CTC, cited in 11% of all original articles on OC (**Figure 2**). Other groups have used custom gene panels suggesting that, apart from *EPCAM*, CKs and mucines, genes such as *ERCC1*, *WT1*, *MAL2*, *LAMB1*, *SERPINE2*, *PPIC*, *TUSC3*, *PGR*, *CDH2*, *ARG2*, *GPX8* and *PRAME* may be valid markers for CTC detection by gene expression analysis [19, 31, 84, 85]. In particular, *PPIC* has been recognized as a particularly efficient OC CTC marker, as it was detected in 70-78% of positive samples [84]. Of note, even though EMT and stem cell markers have been detected in OC CTCs, their qRT-PCR signals were obtained also in healthy donor blood samples [62], indicating they are an inappropriate choice when analysing samples in which leukocytes persist after the enrichment step. In general, when applying qRT-PCR for CTC detection, a rigorous cut-off threshold value is always required to effectively distinguish CTC signals from those of leukocytes [84].

Taken together, while being highly sensitive in simultaneously detecting multiple CTC-derived rare transcripts, gene expression analysis presents with several drawbacks. Similarly to the other marker-based approaches, it requires *a priori* knowledge on the targets of interest and sample lysis for analysis, preventing viable cell collection. Moreover, the approach is not capable of enumeration, and CTC clusters cannot be discriminated from single CTC, leading to the lack of clinically significant information. Finally, if the appropriate controls are not included in the analysis, the interpretation of results can be biased by nonspecific amplification of normal sequences closely related to cancer genes [86] or target genes in noncancerous cells [87].

Molecular marker agnostic approaches for CTC detection

With the aim to avoid biased epitope and gene expression analyses, label-free approaches for CTC enrichment and detection are emerging. Importantly, since methods detecting CTC molecular markers most often require destruction of the sample, development of marker agnostic techniques is appealing not only to account for heterogeneity of CTC subpopulations, but also to allow recovery of intact, viable cells. Label-free separation technologies exploit morphologic and biophysical CTCs features, such as cell size, shape, density, mechanics, deformability, hydrodynamics, electrical polarizability/impedance, and magnetic susceptibility [88, 89]. In OC, for example, cells enriched by ISET

microfiltration have been stained with May-Gruenwald-Giemsa and analysed for the following histo-pathological/cyto-morphological criteria: a) anisonucleosis (ratio >0.5), b) nuclei larger than 1-3 calibrated pore sizes of the membrane (i.e. >8-24 μm), c) irregular nuclear borders, d) high nuclei-cytoplasmic ratio, and/or e) presence of three-dimensional sheets [26]. On the other hand, Phillips and colleagues defined CTCs as having greater volume and reduced dry mass density with respect to leukocytes, and designed a high-definition CTC assay, combining brightfield imagery with the quantitative differential interference contrast microscopy to measure cellular dry mass and cellular volume, respectively [33]. An interesting approach has been reported by Choi and co-workers, who discriminated CTCs from other blood cells by using a microcytometer device where label-free direct current impedance of the cells was measured [90]. The system allows direct liquid biopsy processing and has been used on OC spiked samples with 88% recovery rates, although high numbers of tumor cells (100-2000/mL blood) were used for evaluation [90] (**Table 1**). Another marker agnostic method for OC CTC isolation regards Vita-Assay™ functional separation, based on the tendency of cancer cells to invade collagenous matrices via invadopodia. It consists of slides/plates coated with fluorescently labelled cell adhesion matrix scaffold, where density gradient enriched liquid biopsy fraction of mononuclear cells is seeded [91]. Invasive CTCs ingest fluorescent scaffold, whereas non-adherent cells are washed away. Labelled CTCs may be subsequently analysed by flow cytometry, characterized by additional markers, or cultured *in vitro* and *in vivo* [12, 91-95]. Even though the approach is conceptually intriguing, it has not been evaluated by spiking experiments or compared with other CTC isolation methods.

Conclusions

All currently available CTC analysis methods display limitations, and the choice of the approach depends on the objective of the study. When CTCs are used as prognostic markers, which is currently the most frequent case, detection methods that do not necessarily involve isolation are sufficient. Regardless of the marker-bias, both CellSearch® and Adna Test are attractive approaches, since they offer CTC enrichment and detection in one protocol. Most likely, these and similar kits will be upgraded in the near future, on one hand to allow increased number of simultaneously analyzed markers and, on the other, to offer tumor type-specific panels. In OC, it seems that mesenchymal and stem cell markers should be included, but these are often found also in normal blood. Thus, we expect that approaches permitting single cell isolation and characterization, such as DEPArray®, will lead to the identification of novel markers and guide the design of ideal panels to optimize clinical decisions. In parallel, the development of isolation methods which preserve not only CTC viability, but also CTC cluster integrity are of particular importance, such as label-free microfluidic systems with precisely controlled, gentle flow rates that maintain cell function, morphology and structure. In breast cancer and melanoma, the presence of CTC clusters correlate with worse prognosis, highlighting the clinical value of their detection, which in OC has not yet been achieved.

Even though there is currently no approach available which would allow simultaneous enrichment, detection, enumeration and isolation of viable CTCs from OC patients, the emerging technologies suggest this holy grail is not far from being retrieved, most likely with the help of machine learning systems offering the ultimate label-free CTC detection [96].

Acknowledgements

SL and IK drafted the manuscript. AMP, ADI and GG critically revised the manuscript. IK designed the work. The study was funded by the Italian Ministry of University and Research (PRIN 2017 - Prot. 2017N7R2CJ to IK), by Fondazione Del Monte (Prot. 0000543-24/02/2020), and partly by Associazione Italiana per la Ricerca sul Cancro (AIRC grant IG 22921 to GG).

Disclosure of conflict of interest

None.

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