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Review Article

Circulating tumor cells as a “real-time liquid biopsy”: Recent advances and the application in ovarian cancer

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ABSTRACT

Even with the latest advances in technology, the treatment of ovarian cancer remains a big challenge because it is typically diagnosed at advanced stage, is prone to early relapse in spite of aggressive treatment and has an extremely poor prognosis. Circulating tumor cells (CTCs) can be used as a non-invasive “real-time liquid biopsy”, which has shown the value of diagnosis, assessment of prognosis and chemoresistance, and detection of small residual tumors on ovarian cancer. This review article provides an overview on recent research on CTCs in ovarian cancer, with special focus on the clinical application of CTC tests.

Ovarian cancer (OVCA) was the 8th most common diagnosed cancer in Taiwanese women with 1587 newly diagnosed cases in 2018. It is also the 8th most common cause of cancer mortality in Taiwanese women with 624 deaths in 2018 [1]. Even with current advanced diagnostic tools and the accessibility of medical service in Taiwan, more than half of the cases were diagnosed at advanced stage. The prognosis of OVCA is extremely poor, with reported survival rate below 40% [2].

The detection and diagnosis of cancers currently relies on imaging studies and tissue biopsy. Even the most advanced imaging technologies have limitations in detection small lesions or minimal residual tumors. Tissue biopsy remained the golden standard to make a definitive diagnosis of suspicious tissue. However, it is invasive, and some lesion may be in difficult-to-reach location. Also, tissue biopsy may facilitate the spread of the tumor in cases of OVCA. Therefore, it is essential to identify new prognostic biomarkers to improve the management of OVCA.

Circulating tumor cells (CTCs) and CTC tests

CTCs are tumor cells in the peripheral blood of cancer patients, which shed from the tumor mass and then enter the blood circulation [3]. These cells may develop into new tumor foci under appropriate microenvironment after re-entering tissues again from the blood vessels [4]. Although it remains to be clarified about the mechanisms that CTCs migrate from the origin tumor foci through blood vessels to new foci, these cells may play an extremely important role in the process of tumor metastasis, which is the major cause of cancer death [5,6].

The extreme rarity of CTCs, with an estimated number of 1–10 CTCs per mL of whole blood in metastatic cancer patients, constitutes a major obstacle for CTC detection [7]. Therefore, the key issue for CTC tests is how to efficiently isolate and identify CTCs from an abundance of other blood cells which can be achieved by exploiting biological or physiological differences between these cells.

Techniques for CTC test

The CTC test involves several laboratory procedures, including CTC enrichment, CTC identification and downstream analysis. The procedure of CTC enrichment plays a critical role because of the rarity of the CTCs. Current CTC platforms involve enrichment technologies based on at least one physical and/or biological properties of CTCs (Fig. 1). After enrichment, immunofluorescence or reverse-transcription PCR (RT-PCR) is then performed to identify

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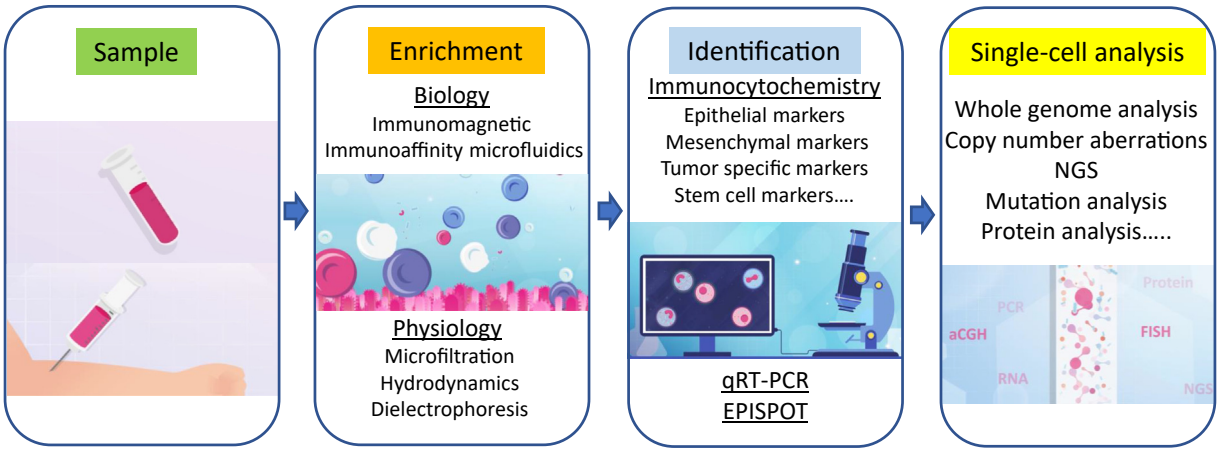

Fig. 1. Schematic of laboratory processes of CTC tests. CTCs in blood of cancer patients can be enriched using physical technique or biological technique. After enrichment, CTCs can be identified by immunocytochemistry staining or other downstream cell analytic techniques.

the CTCs. The techniques involving downstream single cell analysis have also developed.

Biological techniques for CTC tests

Biological techniques involving immunoaffinity approach is the most common method for CTC enrichment. The immunoaffinity technique may positively enrich the CTCs by using epithelial markers or negatively eliminate the blood cells by using hematopoietic markers. Racila et al. first reported the immunoaffinity technique for CTC enrichment [8], and the CellSearch® system, approved by the FDA in 2004, which represents the first-generation Immunomagnetic-based CTC platform for isolating and enumerating CTCs in metastatic of breast, colorectal or prostate cancers [9–11]. The immunomagnetic-based technique uses a magnetic field to separate the CTCs, which are bound with antibody-magnetic bead complexes, from other blood cells.

Immunoaffinity-based microfluidic platform is another biologic technique for CTC enrichment, which is based on microfluidic chips with PEG-biotin-streptavidin layer-coated nanostructure [12,13]. The blood sample is pre-treated with biotinylated antibodies, and the target cells will be captured by the PEG-biotin-streptavidin layer on the microfluidic chip by the interaction between PEG-biotin-streptavidin layer on the nanostructure and the biotinylated antibody on the microvilli of CTCs when the mixed cell suspension flows over the chip.

Epithelial cell adhesion molecule (EpCAM) is a cancer-related antigen, which is usually overexpressed in cancer cells derived from epithelial tissues [14–16]. Anti-EpCAM antibody is therefore used as a major capture antibody to positively enrich CTCs in many immuno'affinity-based CTC techniques. However, the epithelial–mesenchymal transition (EMT) during the process of cancer metastasis may downregulate the EpCAM expression and the EpCAM expression of cancer cells may variate in different cancer type or subtype. EMT is an essential process of cancer metastasis. The cancer cells lose their cell polarity and cell–cell adhesion to become mesenchymal stem cells and gain migratory and invasive properties. The variation in EpCAM expression of cancer cells may greatly affect the capture efficiency of the immunoaffinity-based CTC platform. In the example of epithelial ovarian cancer (EOC), the overall rate of EpCAM overexpression is 73%, while serous adenocarcinoma of ovary has an EpCAM overexpression rate of as low as 55% [17]. Some studies revealed that the combination of epithelial and mesenchymal antibodies can markedly increase the capture rate to overcome the impact of low EpCAM overexpression of CTCs on the capture rate by using single anti-EpCAM antibody [18–21].

Physical (marker-independent) techniques

The physical techniques are based on differences of physical properties between CTCs and non-malignant blood cells, including the cell sizes, densities, electrical charges and deformability. Recently, many studies have developed involving the microfluidic-based physical techniques of microfiltration, hydrodynamics or dielectrophoresis for CTC enrichment [22].

Automation of CTC platforms

In the past few decades, many techniques have been developed to enrich and identify CTCs and microfluidics related CTC platforms may develop the fastest among these technologies [12]. However, all these CTC platforms have not yet been widely used in clinical practice. At present, most of the CTC techniques still rely on much manpower operation, resulting in unstable test results and inability to conduct larger number of tests. Therefore, the automation of the CTC laboratory process has become a key challenge. Some automated systems have recently been developed for CTC detection. Aguilar-Avelar et al. designed high-throughput automated microscopy of CTCs to operate-free and robust analysis of CTCs [23]. Ma et al. also demonstrated an automated immunoaffinity-based CTC platform that can effectively capture SKBR3 Breast cancer cell lines and are used for non-invasive fetal diagnosis [13].

CTC test as a "real-time liquid biopsy"

Advances of automation of CTC platform and high-efficiency microfluidic chips have increasingly demonstrated the possibility of clinical application of the CTC tests. Many studies have exploited the relationship between CTC counts and the progression of disease. Furthermore, the studies on CTCs have gradually progressed from enumeration of CTCs to detailed molecular analyses of the cells and their use as a "real time liquid biopsy" to monitor the progress and the prognosis of the disease as well as the evolution of the tumor cells. The single cell analysis of CTCs allows us to get insights into the molecular characteristics and tumor evolution of the disease and to get more information for decision-making of the management.

Table 1 lists the research on the application of CTC platform to OVCA in the past decade. These studies tried to evaluate the efficiency of diagnosis, the assessment of prognosis and the assessment

Table 1
CTC studies in ovarian cancer (2011–2021).

Author/year/ref.	OVCA case no.	Staging	Enrichment methods	Identification methods/markers	Positive rate (%)	Sensitivity /specificity (%)	Prognostic significance
Microfluidic technique							
Jou HJ (2021) [20]	8	I–III	Immunoaffinity-based, CellReveal system (EpCAM, N-cadherin)	ICC: CD13, EpCAM	62.5	62.5/100	NA
Guo Y-X (2018) [42]	30	I–IV	Size-based microfluidic separation	ICC: HE4, panCK, Vimentin	NR	73.3/63.0	NA
Po JW (2018) [18]	20	Advanced	Immunomagnetic based, Isoflux system (EpCAM, N-cadherin)	ICC: CK, N-cadherin, VE-cadherin, vimentin	90	NA	NA
Rao Q (2017) [52]	23	I–IV	Immunomagnetic based, IsoFlux system (EpCAM)	ICC: EpCAM, Hoechst	87.0	87.0/100	NA
Lee M (2017) [21]	54	I–IV	Biotin-doped Ppy-deposited Microfluidic Device (5 antibodies)	ICC: EpCAM	98%	NA	PFS
Immunomagnetic technique							
Banys-Paluchowski M (2020) [24]	43	I–IV	CellSearch® system (EpCAM)	ICC: CK	26	NA	OS, PFS
Abreu M (2020) [25]	38	I–IV	CellSearch® system (EpCAM)	ICC: CK; RT-PCR: 7 markers	21	NA	NS
Lou E (2019) [26]	23	I–IV	CellSearch® system (EpCAM)	ICC: EpCAM, PanCK	20	NA	NS
Lou E (2018) [27]	35	I–IV	CellSearch® system (EpCAM)	ICC: CK	25.7	25.7/100	NA
Obermayr E (2017) [28]	102	II–IV	CellSearch® system (EpCAM)	ICC: CK7/18, CK5/8 or OvCa (multiple-markers)	26.5	NA	OS (optimally debulked patients)
Liu JF (2013) [31]	78	I–IV	CellSearch® system (EpCAM)	ICC: EpCAM, CK	60	NA	NS
Behbakht K (2011) [29]	54	persistent /recurrent	CellSearch® system (EpCAM)	ICC: EpCAM, CK	NA	NA	NS
Poveda A (2011) [30]	216	Advanced	CellSearch® system (EpCAM)	ICC: PanCK	14.4	NA	PFS, OS
Buderath P (2019) [45]	83	NA	AdnaTest system	RT-PCR: 4 markers	NA	NA	PFS, OS
Chebouti I (2017) [32]	95	I–IV	AdnaTest system (EpCAM)	multiplex RT-PCR: 6 markers	18	NA	PFS, OS
Chebouti I (2017) [33]	65	I–IV	AdnaTest system (EpCAM, MUC1, CA-125)	RT-PCR: EpCAM, MUC-1, CA-125, ERCC1	17	NA	PFS, OS, platinum resistance
Blassl C (2016) [34]	10	NA	AdnaTest system (EpCAM, MUC1)	ICC: EpCAM+/DAPI+/CD45−	30	NA	NA
Kuhlmann JD (2014) [35]	143	I–IV	AdnaTest system (EpCAM, MUC1)	RT-PCR: 4 markers	14	NA	PFS, OS, platinum resistance
Aktas B (2011) [36]	122	I–IV	AdnaTest system (HER2, MUC1)	RT-PCR: 3 markers	19	NA	OS
Zhang X (2018) [46]	109	I–IV	immunomagnetic beads (EpCAM, HER2, MUC1)	ICC: WT1, PAX8, multiplex RT-PCR: 6 markers	90	NA	Chemoresistance, OS
Pearl ML (2015) [40]	123	I–V	CAM uptake enrichment	ICC: EpCAM, MUC16, CD44, FAP qRT-PCR: 4 markers	85.3	83/97	OS
Pearl ML (2014) [41]	88	I–V	CAM uptake enrichment	ICC: CAM, Epi	83.0	83.0/95.1	OS, PFS
Ning N (2014) [53]	21	III–IV	Immunomagnetic beads (CD45)	ICC + FISH: CEP8, CK	76.2	NA	NA
Magnetic separation (folic acid)							
Nie L (2018) [54]	20	NA	Biotin-BSA-FA	ICC: HE4	80	80/100	NA
Liu W (2016) [55]	10	Metastatic	IO-FA nanoparticles	ICC: HE4 and FITC-AffiniPure	50	NA	NA
Size-based technique							
Yang J (2021) [47]	152	I–IV	CanPatrol system	RNA-ISH: EpCAM, CK8/18/19 or Vimentin, Twist	NA	NA	OS
Kim M (2019) [43]	30	I–IV	Tapered-slit filter (TSF) platform	ICC: EpCAM, CK9	76.7	NA	NS
Suh DH (2017) [39]	44	I–IV	Tapered-slit filter (PSF) platform	ICC: CK, EpCAM	77.4	77.4/55.8	NA
Kolostova K (2016) [56]	56	NA	MetaCell system	ICC: WT1, qPCR: 13 markers	58	NA	NA
Kolostova K (2015) [57]	118	I–IV	MetaCell system	ICC: May-Grundwald, Celltracker, NucBlue; qPCR: 5 markers	65.2	NA	NA
Gradient centrifugation							
Kumar J (2019) [48]	37	advanced HGSOC	Gradient centrifugation	ICC: CK, WT1; qPCR: 4 markers	NA	NA	OS
Obermayr E (2013) [44]	200	II–IV	Gradient centrifugation	RT-qPCR: 12 markers	24.5%	NA	NS

OVCA: ovarian cancer; HGSOC: high grade serous ovarian cancer; ICC: immunocytochemistry; NA: not available; NS: not significant; OS: overall survival; PFS: progress-free survival.

of resistance to chemotherapy of OVCA by using CTC enumeration or even single cell analysis of CTC tests.

Immunomagnetic technique is the most frequently used CTC detection method among these studies, including the CellSearch® system in eight studies and the AdnaTest system in four studies. However, research using microfluidic chip technology have recently increased.

Diagnostic value of CTCs on OVCA

Seven studies using the CellSearch® system reported the CTC detection rate but only one study reported the sensitivity and specificity. The overall detection rate of CellSearch® system is quite low on patients with OVCA with a range between 14.4 and 26% [24–30]. Only Liu et al. reported a detection rate up of to 60% in

stage II–IV OVCA [31]. The researches based on the AdnaTest system, a CTC detection platform that combines immunomagnetic technology and RT-PCR technology, also revealed a low detection rate between 14 and 30% [32–36]. The low detection rates may be due to the limitations of the system itself. It may also be due to the fact that hematologic spread may be not the major route of metastasis in case of EOC, therefore not enough CTCs can be captured in the patient's peripheral blood especially in the early stage of the disease [37,38].

Some other studies have used variant CTC technologies to achieve a high positive rate with high specificity and sensitivity of CTCs detection in patients with EOC. However, the case number is limited in most of these studies, and they also couldn't demonstrate the efficiency of early cancer detection. Suh et al. evaluated CTC detection in differential diagnosis of 87 women with adnexal masses by using a size-based CTC technology and found a sensitivity of 77.4% and a specificity of 55.8%. The sensitivity of CTC test for benign tumor vs early stage EOC was 100% [39]. Pearl et al. studied the detection of CTC in 123 EOC patients and revealed a 97% specificity and 83% sensitivity by using an iCTC flow cytometry assay [40]. Another study by the same group demonstrated a 41.2% sensitivity and 95.1% specificity for stage I-II stage EOC [41]. Our previous study also demonstrated that the presence of CD13 markers (a functional cancer stem cell marker) in CTCs has a high positive predictive value in some subtypes of EOC [20]. Guo et al. used a size-based microfluidic platform to detect HE4 + CTCs and compare the sensitivity with CA125 for EOC patients. Their results showed CTC test (73%) had a higher sensitivity than CA 125 (56.7%) [42].

Prognostic value of CTCs on OVCA

Many studies have tried to explore the relationship between the detection of CTCs and the prognosis of EOC. Although some studies failed to show the correlation between the presence of CTCs in blood and the prognosis of EOC. [25,26,29,31,43,44], other studies provided clear evidence that increased CTCs are related to worse overall survival (OS) and/or progress-free survival (PFS) [21,24,28,30,34–36,40,41,45–48].

A recent meta-analysis assessed two clinical trials and thirteen retrospective studies with a total of 1285 patients being included [49]. It revealed that CTC status is significant prognostic indicator for OVCA (OS: HR = 1.77, 95% CI:1.42–2.21, $p < 0.00001$; PFS: HR = 1.53, 95% CI:1.26–1.86, $p < 0.0001$). Regarding enrichment method, it showed that CTCs significantly related to OS either using physical enrichment method (HR = 1.94, 95% CI:1.21–3.09, $p = 0.006$) or using Immunological enrichment method (HR = 1.84, 95% CI:1.37–2.48, $p < 0.0001$).

Molecular characterization or genetic analysis of CTCs may provide further information as prognostic indicators. Gonzalez et al. used multiparametric mass cytometry (CyTOF) to perform in-depth single cell phenotype characterization for high grade serous OVCA and found that patients with poor prognosis had a higher rate to co-express vimentin, HE4, and cMyc cells [50]. Other studies also indicated that the negative prognostic impact of CTC might arise from some special phenotypes associated with treatment resistance, including the presence of ERCC1, cyclophilin C gene, Twist, or PI3Kα [28,33,35]. A recent study by Yang et al. on 152 EOC patients demonstrated that both CTC counts and mesenchymal CTC were independent factors for recurrence [47].

Platinum resistance

Platinum-based drugs are the backbone of systemic treatment of OVCA. Platinum resistance is associated with persistence or early relapse of the disease and failure of the treatment. CTC characterization may serve as a novel tool in the assessment of possible platinum resistance.

The studies by Kuhlmann et al. and Chebouti et al. have demonstrated that the presence of ERCC1-positve CTCs is associated with platinum-resistance in OVCA and is also related to poor prognosis [33,35]. Obermayr et al. have revealed that patients with platinum-resistant OVCA have more detectable CTCs with the gene expression of Cyclophilin C when compared to the patients of platinum-sensitive [28]. A study by Lee et al. showed that the detection of CTC clusters correlated to platinum resistance [21].

Minimal residual disease (MRD)

Another important issue of cancer management is to determine whether further treatment is required after optimal tumor debulking. The current decision-making for additional adjuvant systemic treatment is usually based on the cancer staging and other risk factors. However, the lack of a reliable biomarker to detect MRD or micro-metastasis usually makes the decision-making difficult and may lead to under- or over-treatment of the disease [51]. The detection and elimination of MRD in patients with EOC remains one of the main challenges in gynecologic oncology. CTCs test is supposed to have the potential of a sensitive and specific marker to detect MRD that cannot be detected by current biomarkers or the most advanced imaging modalities.

Obermayr et al. have demonstrated that the detection of CTCs in optimally debulked OVCA patients by the multi-marker protein panel and/or MECOM/HHLA1 FISH had a significantly shorter OS, which represents the existence of MRD or micro-metastasis [28]. Chebouti et al. also reported that patients with MRD after primary cytoreduction surgery had a higher incidence to have detectable EMT-like CTCs [32].

Conclusions

Recent studies have revealed that CTC tests, including CTC enumeration and molecular characterization, may provide non-invasive alternative biomarker for clinical therapy stratification and as a “real time liquid biopsy”, which can be helpful in every step of the management pf cancer patients from diagnosis, assessment of prognosis, evaluation of therapeutic effect, to the prediction of treatment resistance and detection of MRD. Advances in single cell analysis, including profiling of proteomics and genomic aberrations of the CTCs, help us learn more about the mechanisms of cancer metastasis, treatment resistance and cancer evolution.

Even with the promising results of many previous studies, the current clinical guidelines for OVCA still do not support the routine application of CTC tests. More clinical validation is still needed before the liberal use of CTC tests. However, CTC tests may add additional clinical information, in particular the cell markers for treatment resistance or therapeutic targets, which is not available by current diagnostic technologies, and it will show a bright future for personalized medicine.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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