Original Article

Detection of circulating tumor cells and evaluation of epithelial-mesenchymal transition patterns of circulating tumor cells in ovarian cancer

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Background: Circulating tumor cells (CTCs) have considered to be promising liquid biopsy in cancer due to the intact information of whole cells and the potential to reflect micrometastasis. However, CTCs research are extremely limited in ovarian cancer, probably due to their rarity. The predictive value of CTCs and circulating tumor microemboli (CTM) in metastasis remains to be elucidated in ovarian cancer. This study tried to identify CTCs/CTM in ovarian cancer with considerably positive rate. To preliminarily identify the invasive capacity of CTCs/CTM, the epithelial-mesenchymal transition (EMT) patterns of CTCs/CTM was evaluated. Moreover, for comprehensive understanding of invasiveness of disseminated cells in ovarian cancer, EMT pattern of exfoliated tumor cells in ascites were also confirmed in this study.

Methods: Peripheral blood samples and ascites samples were collected from 22 ovarian cancer patients. The Microfiltration combined with morphological analysis was used to detect CTC single cells or cell clusters. Microfiltration combined with morphological analysis was applied in the CTC isolation and identification. EMT was evaluated by immunofluorescence via markers including vimentin and cytokeratin.

Results: Microfiltration combined with morphological analysis was introduced to detect CTCs/CTM with a positivity rate of 40.9% in ovarian cancer patients. The number of CTC varied from 1 to 8, with CTM number from 4 to 30. CTCs/CTM of all samples have experienced EMT process. Vimentin was expressed in all CTC samples and all tumor cells in ascites, while cytokeratin was expressed in 44.4% (4/9) of CTC samples. There were no significant differences of the clinical parameters between the CTC-positive and CTC-negative patients.

Conclusions: This study showed that both CTCs/CTM and detached tumor cells in ascites might have undergone complete or partial EMT in ovarian cancer. Moreover, microfiltration combined with cytomorphological analysis showed a considerable CTC detection rate.

Keywords: Ovarian neoplasms; circulating tumor cells (CTCs); epithelial-mesenchymal transition; metastasis

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Introduction

Ovarian cancer is known as the silent killer, with the highest mortality rate among female urogenital neoplasms. It is estimated that there will be approximately 21,410 new cases and 13,770 new deaths of ovarian cancer in the United States (1). The five-year survival rate for stage I (FIGO) is approximately 80%, but 75% patients have entered stage III or IV at the time of diagnosis, with less than 30% five-year survival rate (2). About 85% of patients still go through pelvic-abdominal recurrence and final death during treatment, even if the disease was completely relieved through the initial treatment (3). To improve the survival benefits, more effective methods should be developed and evaluated for early diagnosis, progression monitoring, drug resistance surveillance and recurrence detection during the disease management (4).

As an emerging minimal-invasive examination, liquid biopsy shows a great promise in precision oncology with its potential to dynamically reflect tumor progression and flexibly guide treatment in real time (5,6). Due to the intact cell structure and comprehensive information of both genes and proteins, circulating tumor cells (CTCs) have acquired much attention (7,8). Actually, CTCs are malignant single cells or cell clusters released into bloodstream from primary or metastatic lesion during tumor dissemination. The CTC clusters were suggested to possess 23- to 50-fold stronger metastatic ability, which are usually called the circulating tumor microemboli (CTM), consisting of more than 2 CTCs, platelets, fibroblasts and so on (9,10). Lots of works have been done to explore the diagnostic and prognostic value of CTC in ovarian cancer, especially in the evaluation of drug resistance and metastasis (11-15). A recent meta-analysis suggested that CTCs before the treatment implied worse survival outcomes and might be a potential biomarker in ovarian cancer (16).

However, faced with the pressure from the immune system and the fluid shear stress, the survival CTC are only 1–10 cells/mL of blood samples, while the number of white blood cells and red blood cells is 10^6/mL and 10^9/mL, respectively (17). Besides, the heterogeneity of epithelial-mesenchymal transition (EMT) status makes the application of the only epithelial marker-based biochemical technology into a dilemma (18,19). Due to the rarity and heterogeneity of CTC, the detection technology has always been a lasting challenge in the CTC clinical application. Recently, the CTC detection system using copper sulfide nanoparticles through photoacoustic detection can specifically identify CTC in ovarian cancer (20). Besides, a new photoacoustic flow cytometry platform realized vivo detection of CTC within 20 seconds in the bloodstream of melanoma patients, which also provided new thoughts about dynamic and efficient CTC detection (21).

During the process of tumor dissemination and metastasis, EMT is believed to be the initial step, with down-regulation of epithelial markers and up-regulation of mesenchymal markers of tumor cells. This phenotype alteration helps detached tumor cells including CTC to avoid the risk of anoikis and alleviate the tension from microenvironment, with stronger invasion ability. It was suggested that EMT status in CTCs might correlate with metastasis, recurrence and clinical stage of tumor (22-24). Ovarian cancer tumor cells are considered to go through partial or complete EMT, a potential target for the inhibition of metastasis (25,26). Vimentin expression is higher in solid metastases compared to primary carcinomas and effusions from ovarian cancer patients (27). Mesenchymal and intermediate mesenchymal ovarian cancer cells showed higher migration, adhesion and invasion capacities (28,29).

Nevertheless, only a few studies focused on the EMT patterns of CTCs in ovarian cancer. Previous study showed that EMT-like CTCs seem to be selected for platinum-based chemotherapy in ovarian cancer (30). A recent recurrence risk stratification showed the considerable potential of the mesenchymal–CTCs to predict recurrence in ovarian cancer, emphasizing the prognostic value of CTCs undergoing EMT (31). Herein, for exploration of detection method with higher positive rate of CTCs in ovarian cancer, we introduced a novel method combining the microfiltration and morphological analysis for the CTC detection. Moreover, for comprehensive understanding the invasiveness of disseminated tumor cells in ovarian cancer, EMT markers, epithelial marker (Cytokeratin) and mesenchymal marker (Vimentin), were detected in CTCs/CTM in blood and exfoliated tumor cells in ascites. We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-529/rc).

Methods

Patients and samples

The 22 subjects with newly diagnosed or relapsed ovarian cancer planning to receive surgery or chemotherapy in the Obstetrics and Gynecology Hospital of Fudan

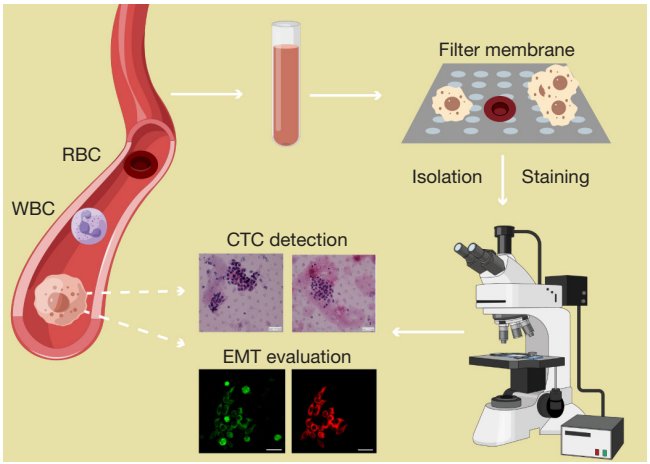

Figure 1 Flow chart of CTCs/CTM enrichment and detection. CTCs, circulating tumor cells; CTM, circulating tumor microemboli; RBC, red blood cells; WBC, white blood cells.

University were included in the study. The collection of the clinicopathological characteristics was performed at the time of enrollment before surgery or chemotherapy, including age, serum CA125 level and so on. About 8 mL of peripheral blood were obtained from 22 patients before the surgery and 10 mL of ascites were obtained from 22 patients during surgery. The Cell Save Preservative Tube (Veridex LLC, Raritan, NJ, USA) were used to store and transport samples from patients at 4 ℃, processed for further analysis within 24 hours.

CTC enrichment and isolation

CTCs were enriched from blood samples by the microfiltration method with the CTCBIOPSY® system (Wuhan YZY Medical Science and Technology Co., Ltd., Wuhan, China) according to the manual instructions (*Figure 1*). The system is based on the ISET platform (isolation by size of tumor cells), with higher CTC positivity than the CellSearch system (32,33). Before loading, 5 mL of the samples were fixed at the room temperature for 10 minutes in the 15-mL centrifuge tubes, containing 3 mL 0.9% physiological saline and 200 μL 0.2% paraformaldehyde (PFA). Then the quality assessment and equipment cleaning of the filtration system were carried out with 75% alcohol and 0.9% physiological saline. The diluted mixture was loaded into wells and then filtered through porous polycarbonate membranes by the positive pressures. The membranes with 8 μm cylindrical pores of the device retain larger and less deformable CTCs while allowing smaller blood cells to pass through, regardless the cell surface protein expression.

CTC detection and characterization

The detection of the CTCs was performed with morphological identification (*Figure 1*). The clusters of at least 3 CTCs were defined as CTM. The membrane with captured CTCs and CTM was taken out and placed on slides. The cells were fixed with 4% PFA for 30 minutes, followed by washing, permeabilization and blocking for further staining. As crucial makers of EMT, the epithelial marker cytokeratin and the mesenchymal marker vimentin were chosen to evaluate EMT in CTCs by immunofluorescence (23,34-36). Antibodies used were including FITC Anti-Cytokeratin antibody (ab52459, abcam) and Alexa Fluor® 647 Anti-Vimentin antibody (ab195878, abcam). After fluorescence microscopy, the cells on the membrane were further stained with hematoxylin and eosin (H&E) for cytomorphological analysis. The isolated cells were microscopically examined by three experienced pathologists to avoid false-positive results. Only cells that appear as large cells with deep stained nuclei and/or atypia, which are features of tumor cells under the microscope, were identified as CTCs. The criteria for the confirmation of CTCs are shown in Table S1 (37).

Statistical analysis

The SPSS 19.0 (SPSS Inc., USA) software was used for statistical analysis. The cell counting was carried out using mean ± standard deviation (mean ± SD). Group differences were analyzed by Fisher's exact test and chi-square test. P value <0.05 was considered significant.

Ethical consideration

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University (No. 2016-48), and written informed consent was taken from all the patients.

Results

Patient characteristics

A total of 22 women aged 36–71 years were enrolled in the study, with pathologically confirmed ovarian cancer

Table 1 Clinicopathological features of 22 patients with ovarian cancer

Parameters	Details	No. (n=22)	No. of CTC/CTM	
			Positive (n=9)	Negative (n=13)
Age, years	≤40	2	1	1
	>40	20	8	12
	Median	54.5 [36–71]		
Stage (FIGO)	II	1	0	1
	III	18	9	9
	IV	2	0	2
	Unknown	1	0	1
Pathologic types	Serous carcinoma	17	7	10
	Clear-cell carcinoma	2	1	1
	Endometrioid carcinoma	1	0	1
	Mucinous carcinoma	1	0	1
	Krukenburg tumor	1	1	0
Platinum response	Sensitive	19	8	11
	Resistant	3	1	2
Lymphatic metastasis	Positive†	5	2	3
	Negative‡	17	7	10
Serum CA125 levels (IU/L)	>1,000	8	3	5
	100–1,000	7	3	4
	<100	4	2	2
	Unknown	3	1	2

†, systematic lymphadenectomy or surgery was performed. ‡, no enlarged lymph nodes were detected in the 17 patients through intraoperative exploration or imaging studies without systematic lymphadenectomy or surgery. CTC, circulating tumor cell; CTM, circulating tumor microemboli.

(FIGO stage II-IV). Among all the patients, 19 (86.4%) were sensitive to platinum and 5 (22.7%) had pathologic lymphatic metastasis. The serum carbohydrate antigen 125 (CA125) level of most patients were at least 100 IU/L when enrolled in the study. The clinicopathological details of CTC-positive patients and CTC-negative patients were shown in *Table 1*.

Isolation and identification of CTC in ovarian cancer

The microfiltration combined with cytomorphological analysis was applied into CTC detection. In total, 40.9% (9/22) of the patients had ≥1 CTCs or CTM; this rate was relatively higher than the CTCs/CTM detection rate reported in previous studies using immunoaffinity-based methods (5). The single CTC number varied from 1 to 8, with CTM number ranging from 4 to 30. Clinical details and CTC numbers of the 9 patients are shown in *Table 2*. The H&E staining results of CTCs/CTM are relatively shown in *Figure 2*. According to the criteria, the CTCs/CTM of ovarian cancer patients exhibited features such as large cells with deep stained nuclei or nuclear atypia under the microscope.

EMT pattern of blood CTCs and ascites disseminated tumor cells in ovarian cancer

To evaluate the EMT pattern of disseminated tumor cells in

Table 2 Clinicopathological features of 9 patients with detected CTCs/CTM

CTCs number	CTM number	Cytokeratin positive	Clinicopathological details					
			Age	Stage (FIGO)	Pathologic types	Platinum response	Lymphatic metastasis	Serum CA125 levels (IU/L)
0	30	4	36	IIIC	Krukenburg tumor	Sensitive	Negative‡	182.5
1	0	0	44	IIIB	Clear-cell carcinoma	Sensitive	Negative‡	40.21
	0	1	48	IIIC	Serous carcinoma	Sensitive	Negative‡	982.6
	0	1	70	IIIC	Serous carcinoma	Sensitive	Negative‡	48.3
	6	0	52	IIIC	Serous carcinoma	Sensitive	Positive†	>1,000
2	0	1	47	IIIC	Serous carcinoma	Resistant	Negative‡	>1,000
3	0	0	46	IIIA	Serous carcinoma	Sensitive	Positive†	>1,000
4	1	0	58	IIIC	Serous carcinoma	Sensitive	Negative‡	745.9
8	0	0	71	IIIC	Serous carcinoma	Sensitive	Negative‡	171.5

†, systematic lymphadenectomy or surgery was performed; ‡, no enlarged lymph nodes were detected through intraoperative exploration or imaging studies without systematic lymphadenectomy or surgery. CTCs, circulating tumor cells; CTM, circulating tumor microemboli.

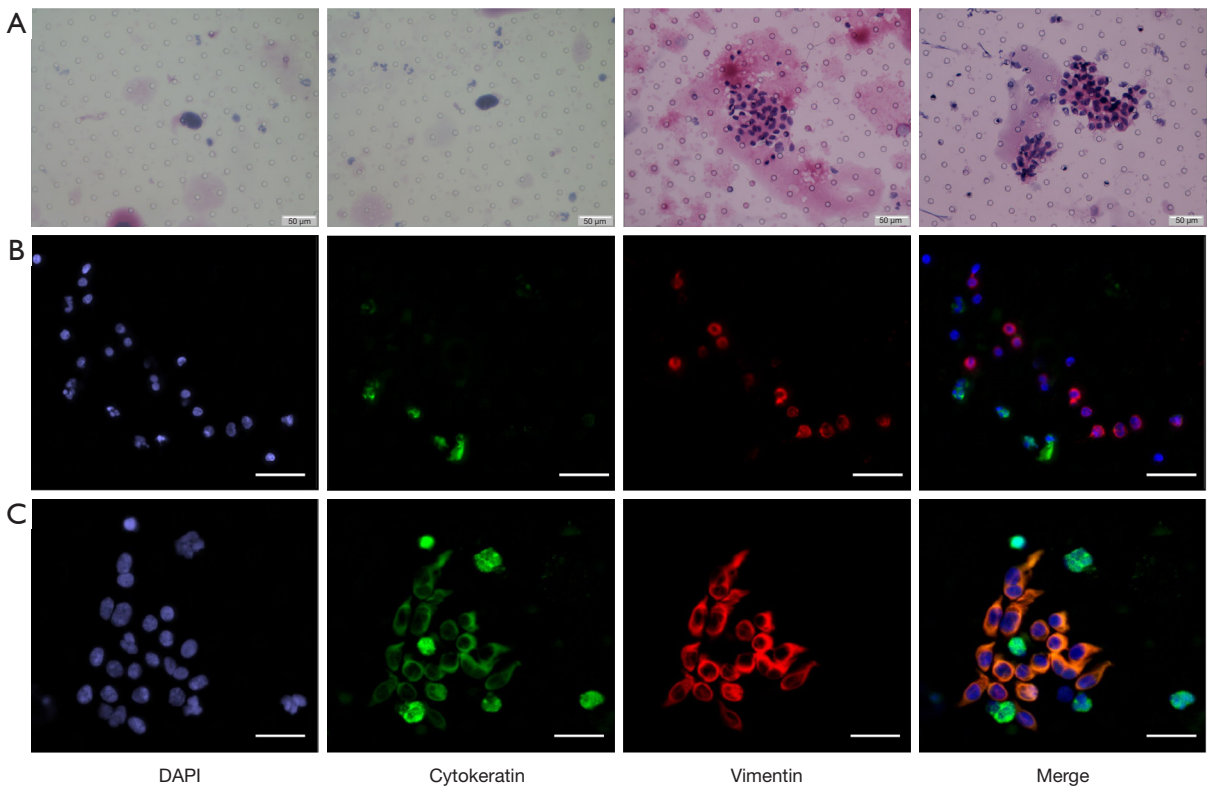

Figure 2 HE staining and immunofluorescent staining of CTCs/CTM. (A) H&E staining of isolated CTCs and CTM. (B) Immunofluorescent staining for vimentin and cytokeratin in CTCs. (C) Immunofluorescent staining for vimentin and cytokeratin in CTM. (Scale bar: 50 μm for HE staining; 50 μm for immunofluorescent staining). DAPI, 4',6-diamidino-2-phenylindole; CTCs, circulating tumor cells; CTM, circulating tumor microemboli; HE, hematoxylin and eosin.

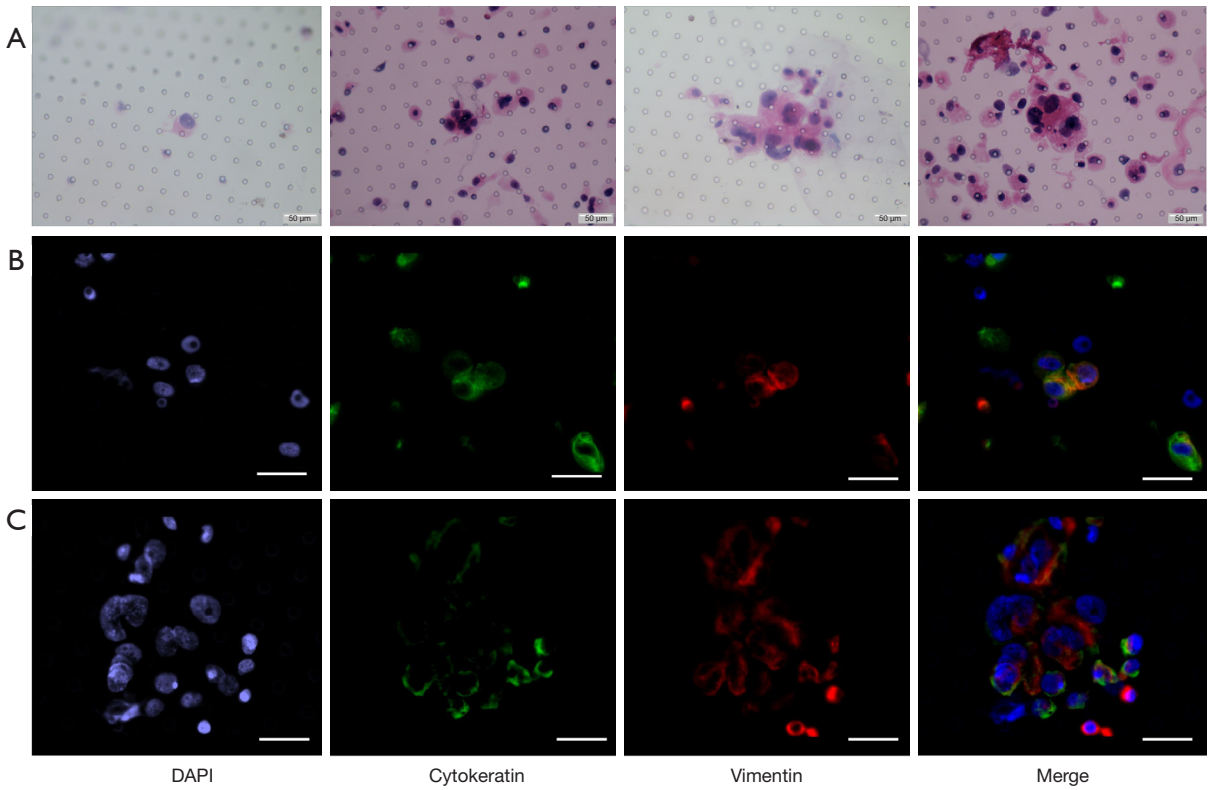

Figure 3 HE staining and immunofluorescent staining of detached tumor cells in ascites. (A) HE staining of isolated tumor cells and cell clusters in ascites. (B) Immunofluorescent staining for vimentin and cytokeratin in disseminated tumor cells in ascites. (C) Immunofluorescent staining for vimentin and cytokeratin in disseminated tumor cell clusters in ascites. (Scale bar: 50 μm for HE staining; 50 μm for immunofluorescent staining). DAPI, 4',6-diamidino-2-phenylindole; HE, hematoxylin and eosin.

blood, vimentin and cytokeratin were immunostained and evaluated in isolated CTCs/CTM in ovarian cancer (*Figure 2*). It was found that vimentin was widely expressed in all samples with detected CTCs/CTM, while cytokeratin was positive in only 44.4% of samples. To evaluate the invasive capacity of disseminated tumor cells in ascites, EMT was also evaluated using cytokeratin and vimentin (*Figure 3*). Similarly, vimentin can be detected in all exfoliated tumor cells in ascites, but cytokeratin is lowly expressed or not expressed. The upregulation of the mesenchymal marker vimentin along with the downregulation of the epithelial marker cytokeratin in CTCs/CTM and tumor cells in ascites indicated that CTCs/CTM disseminated tumor cells in both blood and ascites might have higher invasiveness, with partial or complete EMT.

Preliminary analysis of correlation between CTC and clinical parameters

According to the CTCs/CTM detection, CTC counts were stratified as negative group (no detectable CTC) or positive group (at least 1 detectable CTC or CTM) for analysis of the correlation with clinicopathological variables. Through the subgroup analysis, no significant correlation was found between CTC positivity and characteristics including age, pathologic stage, pathologic type, lymphatic metastasis, platinum response or serum CA125 level. The relationship between the clinicopathological features of patients and the number of CTCs was not analyzed because of the small sample size.

Discussion

Given the potential value of CTC in oncology, the technology of CTC capture and detection increasingly appeared these years, mainly based on the physical properties such as size, density or deformability and biological properties like surface antigens (38,39). The only one FDA-approved platform, the well-known CellSearch

assay, was based on epithelial proteins including epithelial cell adhesion molecule (EpCAM) and cytokeratin. This technology was commonly used in ovarian cancer (40-42). Due to the phenotypic alteration caused by EMT process, the CTCs/CTM with low EpCAM or cytokeratin expression can be missed through the method only using epithelia markers, causing false negatives and inaccurate evaluation of clinical value (43,44). Some studies combined both epithelial and mesenchymal markers to improve the CTC detection rate of ovarian cancer, whereas the combination caused some false positives (45). Recently, methods based on physical properties brought technological progress in CTC studies. The size-based MetaCell platform and some microfluidic devices were applied in CTC capture in ovarian cancer (46,47). These technologies obtained higher CTC positive rate than the immunoaffinity-based methods, avoiding the potential heterogeneity of CTC capture rate caused by EMT process.

Considering the positivity and EMT, our study focused on effective size-based method for CTC studies in ovarian cancer. Among all the physical methods, the ISET filtration technology showed a satisfactory CTC positive rate in gastric cancer (59.32%), non-small cell lung cancer (88%), pancreatic cancer (93%) (37,48,49). Herein, the CTCBIOPSY® platform based on the ISET technology was first applied in CTC detection in ovarian cancer. To avoid the false positives caused by clusters of the blood cells, the cytomorphological verification was combined with ISET. The CTCs/CTM positivity rate in ovarian cancer based on ISET technology is lower than other tumors partly because of the differential metastasis. However, the rate is still higher than most previous studies on ovarian cancer (13,50,51). The result also shows the true positive rate of CTC in ovarian cancer was indeed higher than majority of earlier CTC studies. Besides, the method in our study might provide an alternative way of CTC detection for further studies of CTCs/CTM, using microfiltration combining with cytomorphological analysis. Nevertheless, compared with satisfactory positivity of the tapered slit filter and nanoroughened microfluidic platform, our results should be verified with larger samples (12,52). Hence, more robust combination of enrichment methods and detection technology based on physical or biological traits should be further performed in the CTC evaluation.

To some extent, CTC can help us understand the metastasis mechanism and the genetic information of ovarian cancer, which can be targeted for early diagnosis and treatment. The EMT evaluation of the CTCs/CTM in blood and the tumor cells in ascites in ovarian cancer really contributes to better understanding of hematogenous and peritoneal metastasis of ovarian cancer. In this study, the EMT pattern of disseminated tumor cells was evaluated for further exploration through the detection of vimentin and cytokeratin in 22 ovarian cancer patients. The mesenchymal marker vimentin was expressed in all CTCs/CTM and all tumor cells in ascites, yet epithelial marker cytokeratin in less than half of these cells. This alteration suggests that the detached ovarian cancer cells might have undergone EMT to improve invasion ability during the process of dissemination. Besides, the results also explain the heterogeneous CTC positivity using the CellSearch in ovarian cancer, varying from 14% to 60% in multiple works, as reviewed previously (5). However, only vimentin was used to detect EMT of detached tumor cells in this study. For accurate evaluation of the EMT phenotype of CTCs, E cadherin, N cadherin and transcription factors of EMT should be examined in the future works. As a complex of multiple cells, CTM was suggested to be more aggressive than single CTCs, although it was not elucidated clearly (53). Recent single-cell analysis showed the disseminated tumor cell clusters in the ovarian cancer ascites acquired invasive property via activation of EMT through upregulation of the transcription factor of EMT (28). However, we did not compare the EMT status between CTCs and CTM due to the small sample size.

The existence and heterogeneity of EMT process is believed to participate in cancer invasiveness and dissemination in the early stage of metastasis, contributing to the generation of CTCs/CTM. As reported previously in the correlation analysis, the expression of EMT markers in CTC might be associated with clinical variables such as tumor size, stages and relapse condition (23,54). EMT process can help tumor clusters in ascites possess additional metastasis capacity and drug resistance in ovarian cancer (28). Hence, the inhibition of EMT signaling pathways is expected to block the potential micrometastasis in the initial stage of metastasis, one of the main problems in ovarian cancer. The TGFβ, WNTs, NOTCH and other signaling pathways induce the expression of EMT transcription factors including the zinc finger E-box binding homeobox 2 ZEB, the zinc finger transcription factor SNAIL and the basic helix-loop-helix factor TWIST (55). Inhibitors of stimuli, extracellular mediators and intracellular signaling pathways in EMT have been developed and applicated in cancer treatment (56). Appropriate evaluation of EMT in CTCs/CTM builds the foundation for inhibitors application in

ovarian cancer. To understand the metastasis mechanism, we will also focus on the transcriptional and genomic landscape of disseminated ovarian cancer cells in the future works.

There are some limitations in our study. The sample size and heterogeneous histology of our samples are the main deficiencies. The relatively small sample size makes the relationship between clinicopathological features and CTC positivity in ovarian cancer not conclusive. The comparative analysis of pre- and post-treatment was not carried out in this study. In addition, after cytomorphology and immunofluorescence detection, there were few CTCs remaining for further sequencing or biological property analysis in the next work. In all, further analyzation and verification need to be done by larger sample size.

Conclusions

In this study, we detected CTCs/CTM in ovarian cancer patients using microfiltration combined with cytomorphological analysis, which reduced the risk due to false negatives for CTCs with EMT and false positives due to a cluster of white blood cells and showed a higher detection rate. We also confirmed that the CTCs/CTM and detached tumor cells in ascites exhibited complete or partial EMT, which might reflect the dissemination and micrometastasis of ovarian cancer. Clinical values of CTCs/CTM need to be validated with larger patient cohorts.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-529/rc

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-529/prf

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-529/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-529/coif). CJX reports funding made to the Obstetrics and Gynecology Hospital of Fudan University from the Shanghai Medical Center of Key Programs for Female Reproductive Diseases (grant No. 2017ZZ01016). XYZ reports funding made to the Obstetrics and Gynecology Hospital of Fudan University from the National Natural Science Foundation of China (grant No. 82172747). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University (No. 2016-48), and written informed consent was taken from all the patients.

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