Peer Review File

Article information: http://dx.doi.org/10.21037/tcr-22-529

Reviewer A

Comment1: Overall, it is difficult to understand what this study intends to present through this paper. Please provide a clear purpose about this study, such as whether it is a study that confirms clinical significance or a study that confirms EMT patterns in ovarian cancer.

Reply 1:

Thank you for your advice. One of our purposes is **the latter one** you mentioned. We have modified our Title and Background for presenting a clear purpose for readers (Please see Page1 line1-2; Page1, line 19-22; Page2 line 23-28; Page5, line 101-104; Page13, line 271-275).

Up to now, there are only 60 published CTCs-related articles in ovarian cancer in PUBMED. The relatively limited number in ovarian cancer might be due to the rarity of CTCs in blood and the limitation of detection methods. The primary purpose of this study is identifying CTCs in ovarian cancer with a considerable detection rate.

Moreover, we also tried to confirm EMT patterns of CTCs to evaluate CTCs' prediction value of micrometastasis in ovarian cancer. As for the clinical significance, we believe more CTCs research in ovarian cancer will appear in future.

Changes in the text:

"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. Moreover, the predictive value of CTCs in metastasis remains to be elucidated in ovarian cancer. This study tried to identify CTCs in ovarian cancer and develop CTCs detection methods with higher positive rate. To preliminarily identify the role of CTCs in ovarian cancer metastasis, the epithelial-mesenchymal transition (EMT) patterns of CTCs were also confirmed in this study."

"Introduction: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 confirming EMT patterns of CTCs in ovarian cancer, EMT markers, epithelial cell adhesion molecules (EpCAM) and cytokeratin (CK), were detected in this study."

Comment 2: In this study, vimentin was used as a EMT marker. Recently, however, zeb1 or zeb2 are used as EMT markers because of the problem that vimentin can also be expressed in non CTC-like cells. Please explain reason for choosing vimentin as a EMT marker in this study.

Reply 2:

Thanks for your patient consideration. We have cited some references for the choice of vimentin as EMT marker (Please see Page7, line 137-139).

Three major groups of transcription factors contribute to EMT regulations: the ZEB, Snail and Twist families. As an early trait of EMT, zeb1/zeb2 can bind to the promoter of epithelial marker and

downregulate its expression, which indirectly reflect EMT in cancer(1,2). However, the EMT regulations might be complex and remain to be clear. Moreover, beyond EMT, Zeb1/zeb2 might also regulate multiple non-EMT functions in cancer, mainly including oncogenic transformation, cell stemness, cell cycle, cellular senescence, angiogenesis, hypoxia and immune cell and blood cell development (3-10). Overall, the zeb1/2 expression cannot directly reflect EMT process in cancer and can be disturbed by many elements and noncancer cells(11).

As a highly conservative and stable product of EMT, vimentin has been commonly used as a key marker of EMT in cancer(11-14). Among diverse EMT markers including zeb1/zeb2, vimentin might be the most metastasis-related and prognosis-related one in cancer(11-14). Although vimentin is expressed in some leukocytes, in our study, most blood cells were fileted due to the smaller size and tumor cells were identified with morphological method to keep a relatively high detection rate of CTC.

In future, as we mentioned in the Discussion, combination of multiple EMT markers might be a novel method for CTC identification(15).

Changes in the text:

"As crucial makers of EMT, the epithelial marker cytokeratin and the mesenchymal marker vimentin were chosen to evaluate EMT in CTCs by immunofluorescence."

***Comment 3*:** (p14 Table 1, p15 Table 2) In Table 1, please add the p-value result for each clinical parameter. In table 2, only the total number of CTCs/CTM from the CTC positive sample is presented, please add the number of each heterogeneity CTC (or number of CTCs/CTM which cytokeratin stained).

Reply 3: Thank you for your advice. We have added the number of CTCs/CTM which cytokeratin are positive in table 2(Please see Revised Tables). We did not add P-value in the table due to non-statistical significance as we referred in our text.

Changes in the text: *The highlights in Table 2.*

Comment 4: (p18 Figure 3) Unlike the caption in Figure 3(B), CTCs/CTM appear to coexist in each picture, which making it difficult to distinguish them clearly. Please provide a different figure/capture that is clearly distinguished from each other. And please add a scale bar within the both figures.

Reply 4: Thank you for your advice. We have modified the figure you mentioned and added a scale bar for all figures (Please see new Figure 2 and Figure 3). In addition, for comprehensive understanding of invasive capacity of disseminated tumor cells, we also detected exfoliated tumor cells in ovarian cancer ascites and confirmed EMT patterns of them (Please see new Figure 3, Page1 line26-28; Page 2 line 29,38,42; Page5 line 104; Page6 line 113; Page9 line172-183; Page 11 line 229,234).

Changes in the text: *Please See the new figures and the highlights in context.*

Comment 5: (p19 Supplementary Table S1) Please suggest the references for your CTCs/CTM criteria or data that proves CTCs/CTM was correctly determined through other analysis.

Reply 5: Thank you for your advice. The criteria is according to previous CTCs/CTM research(16). (Page6 line 172)

Changes in the text: *"The criteria for the confirmation of CTCs are shown in Supplementary Table 1(37)."*

Comment 6: Please explain why there are not any control group results like healthy donor blood experiment result during the whole study.

Reply 6: Thank you for your careful consideration. The CTCs detection is not performed in our study due to the relatively expensive cost and the low detection rate of CTCs in normal donors. In addition, the prevalence and patient acceptance of CTCs examination is still limited in China. We believe CTCs might be more prevalent along with the increasement of CTCs researches and deeper identification of its clinical value in cancer.

Reviewer B

The manuscript by Jie et al. showed EMT of circulating tumor cells in ovarian cancer. The authors isolated CTCs by the microfiltration combined with cytomorphological analysis. The isolated CTC and CTM were confirmed with H&E staining. Also, immunefluorescence showed cytokeratin and vimentin positivity in CTCs and CTM. It is important to understand the molecular features of CTC in ovarian cancer; however, a number of data are missing to confirm it. Additional experiments are necessary as shown below.

Comment1: In methods (CTC detection and characterization), no information of vimentin antibody was shown. Please describe all catalogue number of antibodies. Also, the authors mentioned "Epcam" but not data were shown in the results. Please revise this point.

Reply 1: Thank you for your careful consideration. The antibodies we used are FITC Anti-Cytokeratin antibody (ab52459, abcam) and Alexa Fluor® 647 Anti-Vimentin antibody (ab195878, abcam). We have added the antibody information into the method (Please see Page7 line139-142). We have corrected this "Epcam" mistake in our text (Please see Page5 line 102-104; Page 7 line 137-139).

Changes in the text: *"Method: CTC detection and characterization: As crucial makers of EMT, the epithelial marker cytokeratin and the mesenchymal marker vimentin were chosen to evaluate EMT in CTCs by immunofluorescence."*

Comment2: The authors should perform more IF or IHC for verifying EMT in CTCs/CTM. It would be more informative to show ratio of epithelial and mesenchymal cells. For example, E-cadherin and N-cadherin ratio in CTCs/CTM.

Reply 2: Thank you for your advice. We totally agree that the result will be more precise if the ratio of epithelial and mesenchymal cells can be added. However, we did not present the ratio due to the limited sample size of our enrolment, with only 9 CTCs/CTM positive patients and 4 cytokeratin positive patients.

Comment3: If possible, it is better to show FACS sorting data using mesenchymal cell surface markers.

Reply 3: Thank you for your advice. We agree that FACS sorting using multiple mesenchymal markers can improve the accuracy of EMT and EMT heterogeneity(15). However, after HE staining and immunofluorescent staining, few cells remained. FACS sorting was not performed due to the limited number of CTCs/CTMs.

Comment4: In methods, no IRB approval number was shown. Please add it.
Reply 4: This study was approved by the Ethical Committee of Obstetrics and Gynecology Hospital of Fudan University (No. 2016-48). We have added this information into our text (Page10 line 337-338).
Changes in the text: *"This study was approved by the Ethical Committee of Obstetrics and Gynecology Hospital of Fudan University (No. 2016-48)"*.