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Reviewer Comments

Tumor cells undergoing epithelial-mesenchymal transition (EMT) display enhanced ability to enter the circulation, thereby being major source of circulating tumor cells (CTCs). In the manuscript "EMT classification of circulating tumor cells in lung and colon cancer patients: potential role in clinical practice", authors understood the roles of CTC undergoing EMT in monitoring cancer progression.
Couple questions are required to be answered before accepted.

(1) There was similar report (Cancer Biomark. 2017 Dec 6;20(4):487-498) in the PubMed. What is the novel idea in the paper? Please elaborate in the introduction.

Our reply: Thank you for the helpful comments for the further improvement of the manuscript. Although many studies have assessed the clinical relevance of the EMT phenotype of CTC subpopulations in different cancers, the study based on bioinformatic analysis of EMT markers combined with CTC EMT classification has not been reported so far. In this study, by mining TCGA database, we firstly compared the expression pattern of EMT markers between lung cancer and colon cancer. We found that high expression of mesenchymal markers was significantly associated with poor survival of lung cancer patients. However, in colon cancer, most of tumor samples exhibited the hybrid expression spectrum of epithelial and mesenchymal markers.

The new finding based on the bioinformatic analysis promoted us to test whether CTC with mesenchymal phenotype or CTC with hybrid epithelial/mesenchymal phenotypes was an effective biomarker for monitoring tumor progression in lung or colon cancer. We then performed Canpatrol CTC EMT classification assay, and found that the CTC EMT classification data was consistent with the data analyzed by bioinformatics.

Therefore, this is a proof-of-concept study. The novel idea in the paper is the combination of bioinformatic analysis and CTC detection with the EMT classification.

Changes in the text: We have modified our text as advised. (see page 4, paragraph 2, line 7-10 and page 5, paragraph 1, line 1-2)

(2) What is the meaning of "M phenotype" in the abstract?

Our reply: Thank you for the helpful comments for the clarification. The "M phenotype" means "mesenchymal phenotype".

Changes in the text: We have modified our text for the clarification. (see page 2, paragraph 4, line 2)

(3) How to identify the CTC? Please supplement in the introduction. Why to choose lung and colon cancer in the paper? Please supplement in the introduction.

Our reply: Thank you for the helpful comments for the further improvement of the manuscript. The CanPatrol CTC assay was used to enrich and classify CTCs (Wu S et al., PLoS One 2015;10:e0123976.). Peripheral blood samples (5 mL) were collected and lysed by red blood cell lysis buffer to remove erythrocyte, and then the remaining cells were resuspended and fixed in PBS with 4% formaldehyde. The fixed cells were filtered through a membrane with 8 μm diameter pores. The cells on the membrane were analyzed by RNA in situ hybridization (RNA-ISH) to detect CD45 (leukocyte biomarker), EpCAM and CK8/18/19 (epithelial biomarkers), vimentin and Twist (mesenchymal biomarkers). Finally, the samples were stained with DAPI and analyzed with an automated imaging fluorescent microscope.

The CTCs are classified as three subpopulations.

1. CTC with epithelial phenotype (epithelial markers^{+}/mesenchymal markers^{-}/CD45^{-}/DAPI^{+} cells)
2. CTC with mesenchymal phenotype (epithelial marker^{-}/mesenchymal marker^{+}/CD45^{-}/DAPI^{+} cells)
3. CTC with hybrid epithelial/mesenchymal phenotypes (epithelial markers^{+}/mesenchymal markers^{+}/CD45^{-}/DAPI^{+} cells)

Regarding to the question “Why to choose lung and colon cancer in the paper”, the original idea of this study was from a bioinformatic analysis of EMT markers in lung and colon cancers. By mining TCGA database, we firstly analyzed the expression pattern of EMT markers in lung or colon clinical tumor samples. The bioinformatic analysis indicated that mesenchymal markers were expressed in lung tumor samples, and its high expression of mesenchymal markers was significantly associated with patients’ poor survival in lung cancer. However, unlike lung cancer, in colon cancer most of tumor samples exhibited the hybrid expression spectrum of epithelial and mesenchymal markers. Therefore, the new finding based on the bioinformatic analysis promoted us to test whether CTC with mesenchymal phenotype or CTC with hybrid epithelial/mesenchymal phenotype was an effective biomarker for monitoring tumor progression in lung or colon cancer, respectively.

Changes in the text: We have modified our text as advised. (see page 4, paragraph 1, line 2-7 and page 4, paragraph 2)

(4) Please supplement the progress of the prognostic biomarkers for lung and colon cancer in the introduction.

Our reply: Thank you for the helpful comments for the further improvement of the manuscript. Since the manuscript focused on CTC, we supplemented the progress of CTC as the prognostic biomarkers for lung and colon cancer.

Using the CanPatrol CTC EMT classification system, a recent study indicated that lung adenocarcinoma patients with positive mesenchymal CTC had a significantly poor recurrence-free survival and overall survival, compared with the patients with negative mesenchymal CTC (Peng et al. Cancer Manag Res 2020.12:5105-5117). The result is consistent with our study indicating that CTC with mesenchymal phenotype could be an effective biomarker for monitoring tumor progression in lung cancer.

In a study including 299 patients with colon cancer, CTC was detected using CK20 RT-PCR as well as immunocytochemistry staining with anti-pan-keratin and anti-EpCAM antibodies. The study indicated that detection of CTC with CK20 RT-PCR was an independent predictor of worse overall survival and disease free survival in colon cancer (Hinz et al. BMC Cancer 2017. 17(1):53).

Changes in the text: We have modified our text as advised. In order to keep the flow of the presentation, the progress of the prognostic biomarkers for lung and colon cancer was supplemented in the discussion section. (see discussion section, paragraph 2-3)

(5) The case samples were too small. How to handle with the issue?

Our reply: Thank you for the helpful comments for the clarification. Indeed, the case samples were small in our study. From January 2019 to December 2019, a total of 31 patients with lung or colon cancer at the Hefei Cancer Hospital, Chinese Academy of Sciences (Hefei, Anhui, China) were enrolled.

In the study, our idea is from a bioinformatic analysis. Through TCGA data mining, we found that high expression of mesenchymal markers was associated with patients' poor survival in lung cancer. In colon cancer, most of tumor samples exhibited the hybrid expression spectrum of epithelial and mesenchymal markers. The new finding based on the bioinformatic analysis promoted us to test whether CTC with mesenchymal phenotype or CTC with hybrid epithelial/mesenchymal phenotype was an effective biomarker for monitoring tumor progression in lung or colon cancer, respectively. Our data on CTC with EMT classification indicated that CTC with M phenotype in lung cancer, or CTC with hybrid E/M phenotypes in colon cancer appeared to be potent for monitoring tumor progression, which was consistent with the data analyzed by bioinformatics.

Therefore, this is a proof-of-concept study. It is anticipated that a large scale clinical study will be conducted in the future.

Changes in the text: We have modified our text as advised. (see discussion section,

paragraph 4)

(6) In the figure 4 and 5, the scale bar is needed to add. In the figure 4 and 5, what was the red fluorescence standing for (EpCAM, CK8/18/19)? And green fluorescence? Please supplement in the figure 4 and 5 legend.

Our reply: Thank you for the helpful comments for the clarification. The scale bars have been added in the figure 4 and 5.

The probes (for the epithelial biomarkers EpCAM and CK8/18/19) were conjugated with the fluorescent dyes Alexa Fluor 594 (Red fluorescence). The probes (for the mesenchymal biomarkers vimentin and twist) were conjugated with the fluorescent dyes Alexa Fluor 488 (Green fluorescence). Therefore, the red fluorescence stands for the epithelial biomarkers, EpCAM and CK8/18/19. The green fluorescence stands for the mesenchymal biomarkers, Vimentin and Twist.

Changes in the text: We have modified our text as advised. (see page 19, paragraph 2 and page 20, paragraph 2)

(7) In the figure 4 and 5, what was the arrow standing for? Please supplement in the figure 4 and 5 legend.

Our reply: Thank you for the helpful comments for the clarification. The arrows stand for the sites of tumors by CT scanning.

Changes in the text: We have modified our text as advised. (see page 20, paragraph 1 and 2)

(8) Why not to test the expressions of EpCAM, CK8/18/19, Vimentin and Twist in the collected samples?

Our reply: Thank you for the helpful comments for the clarification. In the study, we used the CanPatrol CTC detection system to classify CTCs into three subpopulations (E phenotype, M phenotype and hybrid E/M phenotypes). The mRNA expression of epithelial biomarkers EpCAM and CK8/18/19 are detected by probes conjugated with the fluorescent dyes Alexa Fluor 594 (Red fluorescence). The mRNA expression of mesenchymal biomarkers vimentin and twist are detected by probes conjugated with the fluorescent dyes Alexa Fluor 488 (Green fluorescence). However, the technique can not detect the expression of each marker (EpCAM, CK8/18/19, Vimentin and Twist).

This is a good idea to test the expressions of EpCAM, CK8/18/19, Vimentin and Twist in the collected samples. In the future, we can test the expressions of EpCAM, CK8/18/19, Vimentin and Twist in CTC samples by RT-PCR.

Changes in the text: None.