Peer Review File

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Reviewer #A

The reviewer's general comment*: The authors detected the CTCs from the portal vein (PoV) and peripheral vein (PV) blood and found the distribution of EMT related CTCs and survival impact. This manuscript was well organized, but some issues still need to be answered.*

The authors' response: We sincerely appreciate all of your positive comments.

The reviewer's major comment 1*: Cellsearch or ISET system is commonly used for CTC study. The method in this study for CTC isolation was not appropriate. When using the density gradient centrifugation, the tumor cells may not in the same layer with BPMC or be lost.*

The authors' response: We thank reviewer's kind comment. Yes, we agree with the reviewer's opinion. Cellsearch or ISET system has been commonly used for CTC study because of their own features and advantages. Cellsearch system is primarily intended to isolate epithelial CTCs by EpCAM positive enrichment method (1). While, ISET system is able to isolate the CTCs with larger size than peripheral blood leukocytes using filters with 8μm

diameter circular pores (2).

No matter which CTC isolation method is applied, a pretreatment for blood samples is needed in advance to get rid of red blood cells and leave nucleated cells even by CellSearch or ISET strategies. The method of density gradient centrifugation which we chose to pretreat blood samples has been reported to be an efficient way to separate nucleated cells including CTCs and mononucleocytes fraction away from erythrocytes as the similar way as CellSearch used (3-5). After collected nucleated cells from peripheral blood, normally identify CTCs by different immunomorphological and molecular characterization of the cells. CellSearch uses the positive selection by enrichment of EpCAM positive cells from the mononucleated cells. Weused a different approach to identify the CTCs from mononucleocytes fraction by removing leukocytes (CD45 marker positive), so called "negative selection". Because our research focused on the spatial heterogeneity in EMT properties of CTCs which may include big or small size CTCs. We used density gradient centrifugation to avoid the loss of small size CTC group which was reported to be a crucial indicator for cancer relapse (6). Also, the CellSearch isolation method, which uses EpCAM on the surface of CTCs for cell isolation, did not recognize, in particular, normal-like or small cancer cells, which in general have aggressive features.

Truth is, due to the complexity and heterogeneity of CTC, there is still not any unified method in the field of CTC isolation (3). As a consequence, the most important thing for researchers is to choose an appropriate method according to their study objective and keep this repeatable method throughout the whole study. To clarify it, we modified the text as advised (see Page 5, lines 89-95).

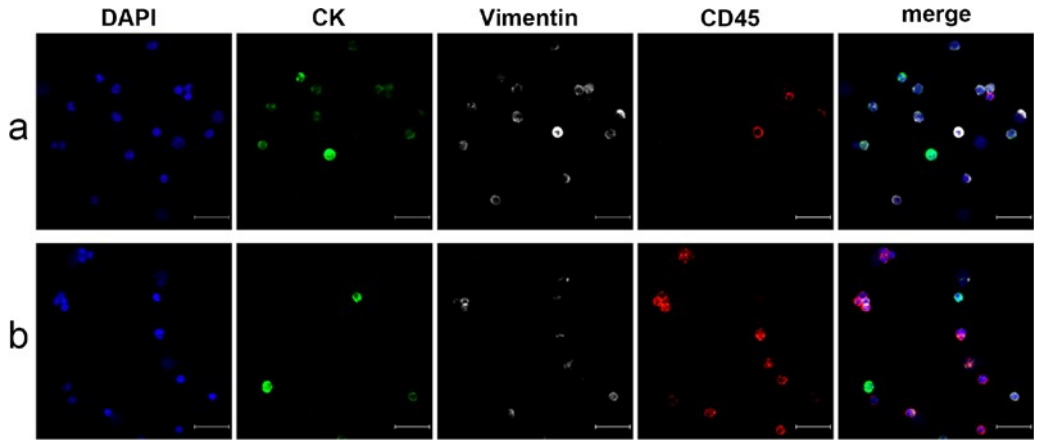
The reviewer's major comment 2*: There will be still so many white blood cells by the CD45 negative enrichment method and detection will be difficult in my experience. How did authors count the CTCs and did they have the scan images?*

The authors' response: We thank reviewer's kind comment.

Currently, there is no known ideal CTC surface antigen target that is capable of capturing all CTCs (3). The CD45 negative enrichment method we used was proved as an alternate approach to positive immunoaffinity basedCTC selection (3, 7, 8).

In our study, we stained cells with multiple immunofluorescence (CK-FITC, vimentin-alexa fluor 647, CD45-PE and DAPI). After scanning, the images were visualized to acquire entire fluorescence signal of each multicolor channel. Identification criteria of positive CTC were $DAPI^{+}/CD45^{-}/CK^{+}$ and/or $vimentin^{+}$ cells. We agree with the reviewer's opinion. There were still some white blood cells by the CD45 negative enrichment assay, but it is OK after scanning and merged images from multicolor channels (please to see the supplementary figure below).

Additionally, CTCs were also could divided into three subtypes based on the staining: 1) epithelial CTCs (E-CTC), CK^{+}/ $Vimentin^{-}$/ $CD45^{-}$/ $DAPI^{+}$;2) epithelial/ mesenchymal hybrid CTCs (E/M-CTC), CK^{+}/ $Vimentin^{+}$/ $CD45^{-}$/ $DAPI^{+}$; 3)



mesenchymal CTCs (M-CTC), CK^{-}/ $Vimentin^{+}$/ $CD45^{-}$/ $DAPI^{+}$.

Figure S1. Multiple fluorescent images containing CTCs and residual white blood cells from the same patient. Images were from the portal blood sample(a) and the peripheral blood sample (b). DAPI, blue; CK, green; Vimentin, white and CD45, red. Scale bar: 30 μm.

According to the reviewer's comment, we modified the text (see Page 7, lines 135-142 and Page 8, 157-158) and added the supplementary figure in the revised version as *Figure S1*.

The reviewer's minor comment 3*: There was limited cases (39), so all indicators couldn't enter into multivariate analysis.*

The authors' response: We appreciate the reviewer's insightful comments and agree with that the sample size is important for further analysis. We spent more than 1 year in recruiting patients. However, the low curative resection rate in pancreatic cancer (less than 20% at initial diagnosis) rendered portal blood samples collection really difficult. We understand that this represents a major limitation of the current study,but such a weakness is probably inherent to many similar studies of CTCs in portal vein (9-17).

References	Year	Study cohort	Sample sizes	Detection methods
Braga et al (9)	2014	Resected pats.	20 PC pats.	EpCAM+ CTCs selection / CellSearcl
Waxman et al (10)	2015	Borderline or Unresectable pats.	14 PC & 4 other pats.	EpCAM+ CTCs selection / CellSearcl
Litherland et al (11)	2016	Resected pats.	21 PC & 20 other pats.	FACS isolation with CD44+, CK19+/CD147+, EpCAM+ CTCs
Lee et al (12)	2016	Resected pats.	42 PC & 24 other pats.	EpCAM+ CTCs selection / CMx
Litherland et al (13)	2018	Resected pats.	11 PC & 30 other pats.	FACS isolation with CD44+, CD147+ EpCAM+ CTCs
Lundholm et al (14)	2018	Resected pats.	13 PC & 4 other pats.	EpCAM+, CKs+ (CK-8, -18, -19) CTC selection / Isoflux
Chen et al (15)	2018	Unresectable pats.	29 PC pats.	CK19+ or EpCAM+ selection / ClearBridge ClearCell FX system
Xiu et al (16)	2019	Resected pats.	24 PC & 15 other pats.	FCM detection with EpCAM+ CTCs
Tzeng et al (17)	2019	Resected pats.	14 PC pats.	EpCAM+ CTCs selection / CellSearcl

PC, pancreatic cancer; Pats., patients;

As the reviewer suggested, we have modified our statistical methods, and differences in RFS between patient groups were tested using the log rank test and hazard ratios were estimated from Cox proportional hazards models that adjusted for age and gender. According to the modified results, we revised the manuscript text (see page 7, lines 150-152; Page 10, 189-197 and 199-205), *Table 2* and *Figure 2*.

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The Editorial Comments 1: *The language of your manuscript needs to be further polished by a native English speaker or some companies that provide the manuscript services.*

The authors' response: We sincerely appreciate all of your positive comments. We have carefully proofread the revised manuscript to minimize the typographical, grammatical, and bibliographical errors. Moreover, the revised manuscript has been edited by American Journal Experts (https://www.aje.com/zh) to further improve the phrasing and readability. The verification code is 8BA7-3EA7-D3C0-2696-4FB5.

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Spatialheterogeneityinepithelialtomesenchymaltransitionpropertiesofcirculating tumorcells associated with distant recurrence in pancreatic cancer patients

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