



Original Articles

Vimentin-positive circulating tumor cells as a biomarker for diagnosis and treatment monitoring in patients with pancreatic cancer



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ABSTRACT

The identification of circulating tumor cells (CTCs) relies on epithelial tumor cell markers. In the present study, we aimed to determine whether cell-surface vimentin could be a biomarker to isolate CTCs in pancreatic ductal adenocarcinoma (PDAC). Vimentin was identified as highly expressed on the surface of mesenchymal-phenotype pancreatic tumor cells. Vimentin⁺ CTCs were detected in 76% of patients with PDAC (76/100) using CTCs enriched via a microfluidic assay. A cut-off value of two vimentin⁺ CTCs distinguished patients with PDAC from healthy individuals. Combined vimentin⁺ CTCs and Carbohydrate antigen 19–9 provided favorable diagnostic potency, with an area under the curve of 0.968. Vimentin⁺ CTCs counts correlated with the change in tumor burden for patients undergoing resection. Significantly reduced CTC counts were observed after chemotherapy in subjects that responded to treatment. Preoperatively higher CTCs counts correlated with shortened recurrence-free survival. Taken together, vimentin⁺ CTCs could be a reliable biomarker in pancreatic cancer. The enrichment of mesenchymal CTCs complements the strategy of capturing epithelial CTCs, allowing a more thorough interrogation of the biology and clinical significance of CTCs in PDAC.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy with poor prognosis [1,2]. Only a small proportion of patients have resectable disease because of the occurrence of metastasis and vascular invasion. Even in patients who undergo curative resection, early recurrence, either locally or in distant organs, is common [3,4]. This suggested that current criteria are not appropriate to select patients who might benefit from surgery. For patients with unresectable disease who receive chemotherapy, useful biomarkers to predict the drug

response are lacking. Although carbohydrate antigen 19–9 (CA19-9) has been used frequently in clinical practice, it has been suggested that it does not confer additional value in guiding treatment. Besides, CA19-9 is not appropriate in those patients that are genetically incapable of producing the Lewis antigen [5]. Thus, more reliable biomarkers that can aid decision-making during diagnosis and treatment are urgently needed [6].

Circulating tumor cells (CTCs) refer to malignant cells that are shed from the primary tumor into the vasculature, which are believed to be the direct source of distant metastasis [7]. Accumulating evidence

Abbreviations: CTC, Circulating tumor cells; PDAC, Pancreatic ductal adenocarcinoma; EpCAM, Epithelial cell adhesion molecule; EMT, Epithelial-mesenchymal transition; IPMN, Intraductal papillary mucinous neoplasm; CK, Cytokeratin; CA19-9, Carbohydrate antigen 19–9; CAFs, Cancer-associated fibroblasts; TGF- β , Transforming growth factor beta

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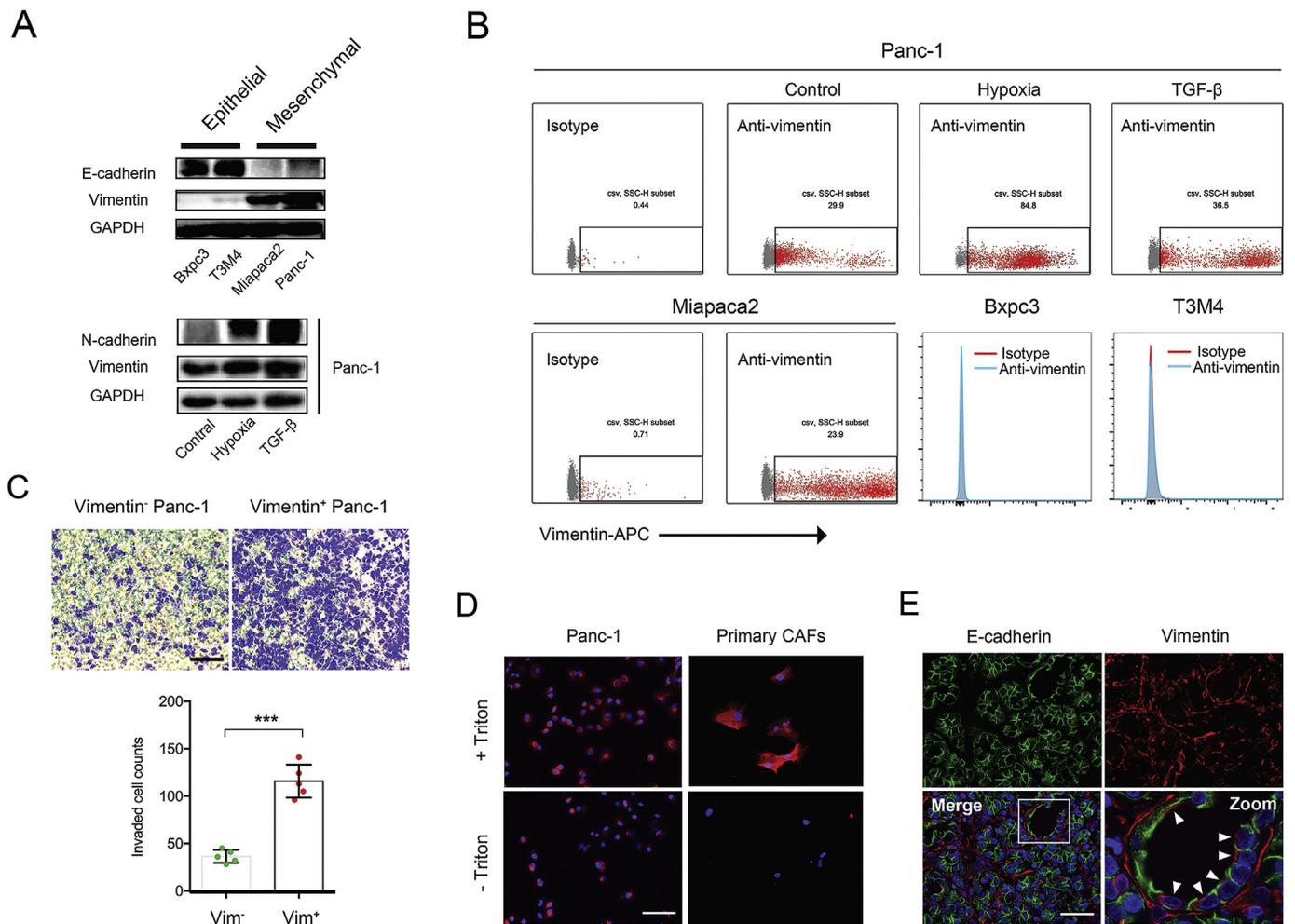


Fig. 1. Validation of the cell-surface vimentin as a marker for mesenchymal pancreatic tumor cells. Immunoblotting (A) and flow cytometry (B) analysis for vimentin in epithelial and mesenchymal pancreatic cell lines. Comparison of the migration of sorted vimentin⁺ and vimentin⁻ PANC-1 cells (C), the scale bar represents 50 μ m. Three biological replicates were performed. Immunofluorescence staining for vimentin in PANC-1 and primary cancer-associated fibroblasts (CAFs) (D); the scale bar represents 25 μ m. Immunofluorescence staining for vimentin and E-cadherin in a pancreatic cancer tissue (E); the scale bar represents 50 μ m ****p* < 0.001.

confirms that CTCs have promise in a wide range of applications, including diagnosis, tumor staging, disease monitoring, and identification of therapeutic targets [8–10]. In breast cancer, CTC counting has been included in the latest version of the National Comprehensive Cancer Network guidelines and is defined as a prognostic factor in the American Joint Committee on Cancer staging. Although the evidence is not so strong in pancreatic cancer, a report has suggested that CTCs could be a useful biomarker in this malignancy [11].

To date, the majority of studies have used epithelial cell adhesion molecule (EpCAM), an epithelial cell marker, to identify CTCs. As the first and only FDA-approved CTC-detection platform, CellSearch[®] is based on an immunomagnetic method to capture EpCAM⁺ CTCs. In a study evaluating the efficiency of CellSearch[®] for 964 patients across 12 different types of metastatic cancers, PDAC patients had the lowest detection rate and CTC counts [12]. Similarly, other studies adopting the CellSearch[®] pipeline reported low detection rates of CTCs in PDAC (6–30%) [13–15]. In contrast, enrichment of EpCAM⁺ CTCs using microfluidics approaches, such as the ¹HbCTC (herringbone)-Chip, showed favorable detection sensitivity. In addition, a few studies reported better detection efficiency of isolated CTCs using size-based filtration compared with that achieved using CellSearch[®] [16]. In addition, EpCAM-based identification of CTCs has been challenged for years because it does not fit logically with the theory of epithelial-mesenchymal transition (EMT), which is believed to be an essential step of tumor metastasis [17,18]. Theoretically, CTCs are more likely to have a

mesenchymal phenotype because cells that have undergone EMT have enhanced capacities of migration and invasion. Hence, using EpCAM might underestimate the number of CTCs by missing EpCAM⁻ CTCs, which probably have a mesenchymal phenotype and are critical for distant metastasis. Clinical sample analysis confirmed that EMT in primary tumors was associated with increased risk of metastasis [19]. A lineage tracing study in a genetically engineering mouse model suggested that pancreatic tumor cells are disseminated via EMT as early as the pre-cancerous stage [20]. These results supported the view that detection of CTCs with a mesenchymal phenotype using suitable markers in patients with PDAC is not only possible, but also important. Although a small number of studies have examined the mesenchymal phenotype of CTCs, in these studies, the initial CTC enrichment relied on epithelial markers [21–23]. Thus, the accurate status of CTCs with a mesenchymal phenotype remains enigmatic.

Vimentin is a typical marker that is overexpressed during EMT. Vimentin can be located on the tumor cell surface in sarcoma, breast, and colon cancer [24]. This raises the possibility that cell-surface vimentin could be a potential marker to identify mesenchymal CTCs. In the present study, we applied a microfluidic chip-based platform to enrich and identify cell-surface vimentin-positive CTCs in pancreatic cancer, and analyzed their clinical significance.

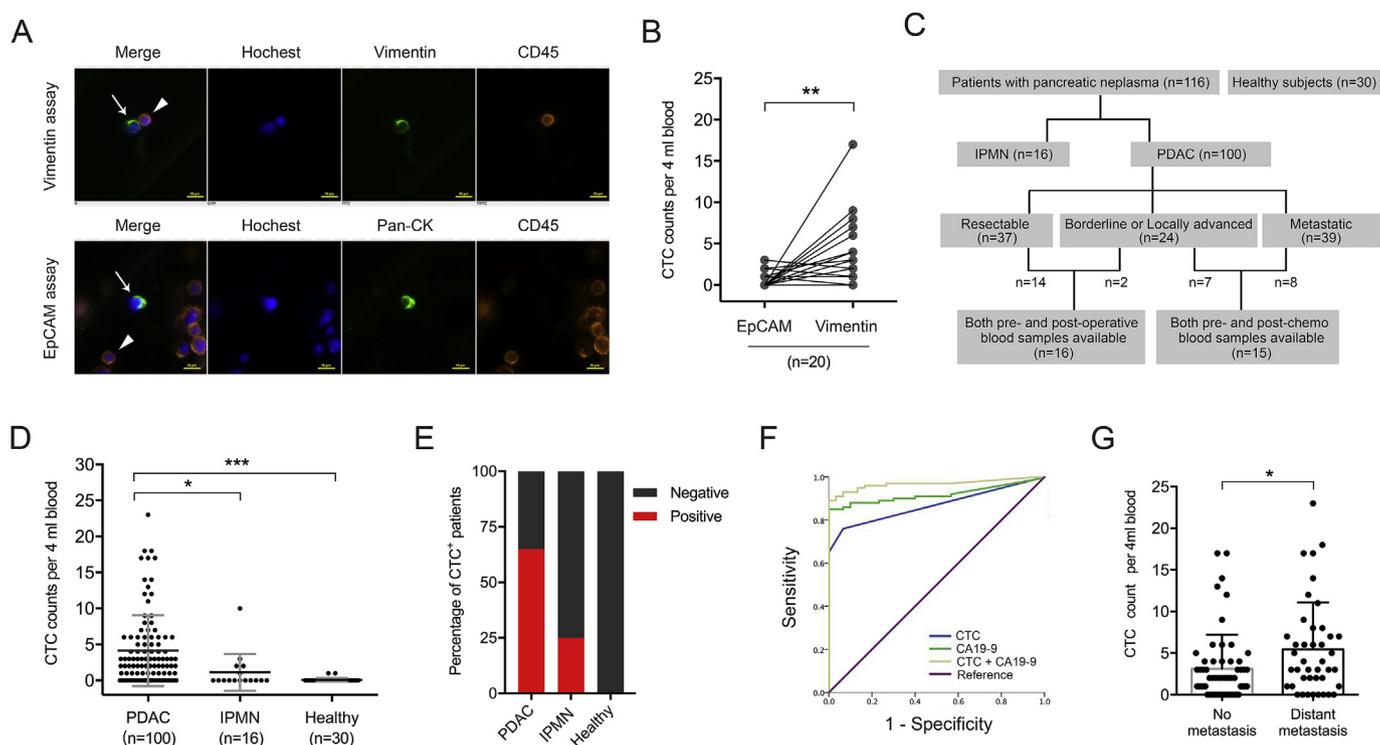


Fig. 2. Vimentin⁺ circulating tumor cells (CTCs) constitute a potential biomarker in pancreatic cancer. The identification of vimentin antibody or epithelial cell adhesion molecule (EpCAM) antibody-enriched CTCs (A); arrows indicate CTCs, and arrowheads indicate white blood cells. The paired comparison of CTC detection using vimentin versus the EpCAM assay (B). Flowchart of enrolled patients and blood sample analysis (C). The detection of vimentin⁺ CTCs in patients with pancreatic ductal adenocarcinoma (PDAC), intraductal papillary mucinous neoplasm (IPMN), and in healthy subjects (D). A cut-off value of two cells/4 ml of blood was used to discriminate positive and negative CTC detection (E). The diagnostic performance of vimentin⁺ CTCs, carbohydrate antigen 19–9 (CA19-9), and combined vimentin⁺ CTCs with CA19-9 was shown by a receiver operating characteristic (ROC) curve (F). Comparison of CTC counts for patients with or without distant metastasis (G). **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

2. Materials and methods

2.1. Study design and participants

The study was conducted according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria [25]. In total, 100 patients with PDAC, 16 patients with intraductal papillary mucinous neoplasm (IPMN), and 30 healthy individuals were enrolled. The included participants were all consecutive and diagnosed between December 2016 and November 2017. Written consent for the blood and tissue sampling was obtained from each patient and healthy donor. All included patients with PDAC had negative history of malignancy, and were treatment-naïve before enrollment. Diagnosis of unresectable PDAC was supported by the pathological results of biopsy of either the primary tumor or metastatic lesions. Healthy individuals had no medical history of any malignant disease. The patient demographics and clinical information included age, gender, serum CA19-9 levels, tumor location, stage, and treatment choice. For patients who underwent surgical resection, other information, including tumor size, grade, perineural and perivascular invasion, and nodal and margin status were further collected. Assessment of chemotherapy response was performed via abdominal enhanced computed tomography and enhanced magnetic resonance imaging. According to the Response Evaluation Criteria in Solid Tumors version 1.1, measurement of the tumor response by imaging was qualitatively classified into four categories: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).

Blood samples were obtained after the approval of the Institutional Review Board at the Second Affiliated Hospital of Zhejiang University, School of Medicine. Peripheral blood (5–10 ml) was harvested in EDTA-pretreated blood collection tubes. In a subset of patients, portal venous

blood was drawn intraoperatively before and after removal of pancreatic cancer. This work was conducted in accordance with the guidelines of the Declaration of Helsinki.

2.2. CTCs enrichment and enumeration

Blood samples were processed within 6 h of collection. Peripheral blood mononuclear cells (PBMCs) were first acquired via density gradient centrifugation. The PBMC fraction was then washed and loaded into a CytoQuest™ CR system. Potential CTCs were captured using a vimentin or EpCAM immobilized microfluidic chip. Immunofluorescence staining was performed using fluorescein isothiocyanate (FITC) anti-Pan-cytokeratin (CK) and FITC anti-vimentin antibodies for EpCAM- and vimentin-captured cells, respectively. Phycoerythrin (PE) anti-CD45 antibodies were used to label white blood cells, while nuclei were marked using Hoechst staining. The chip slides were scanned using automated fluorescence microscope equipment (Nikon Ti-E). The imaging results were then analyzed independently by two experienced technicians. CTCs were defined as vimentin⁺CD45⁻Hoechst⁺ or Pan-CK⁺CD45⁻Hoechst⁺.

2.3. Cell lines

Human pancreatic cell lines PANC-1, MIA PaCa-2, BxPC-3, and T3M4 were obtained from the ATCC (Manassas, VA, USA). All cell lines were authenticated using a short tandem repeat (STR) DNA test. A primary cancer-associated fibroblasts (CAFs) line was generated and preserved in our laboratory from a fresh sample for one patient with PDAC under written informed consent for research purposes.

Table 1
Clinicopathological characteristics of patients with ≥ 2 or < 2 vimentin⁺ CTCs.

Parameters	Total (n = 100)	≥ 2 CTCs (n = 65)	< 2 CTCs (n = 35)	p value
<i>All patients (n = 100)</i>				
Age, median (range, years)	64 (43–90)	63	65	
Gender				0.199
Male	60	36	24	
Female	40	29	11	
Tumor location				0.377
Proximal	54	33	21	
Distal	46	32	14	
AJCC staging				0.015
I	8	6	2	
II	31	17	14	
III	22	10	12	
IV	39	32	7	
Metastasis				0.005
Liver	28	24	4	
Other	13	10	3	
CA19-9, median (range, U/ml)	2439.8 (2–12000)	2866.4	1647.6	0.145
CA19-9 level				0.852
Low (< 36 U/ml)	19	12	7	
High (> 36 U/ml)	81	53	28	
<i>Patients undergoing resection (n = 40)</i>				
Tumor grade				0.031
Well/moderate	18	7	11	
Poor	22	16	6	
Perineural invasion				0.397
Positive	35	21	14	
Negative	5	2	3	
Microvascular invasion				0.385
Positive	22	14	8	
Negative	18	9	9	
Lymph nodes status				0.272
Positive	26	13	13	
Negative	14	10	4	
Tumor size, mean (cm)	3.2	3.4	3.0	0.304

CTC, circulating tumor cells; CA19-9, carbohydrate antigen 19-9; AJCC, American Joint Committee on Cancer.

2.4. Flow cytometry analysis

Cultured cells were detached using non-enzymatic dissociation buffer and stained with anti-vimentin antibodies for 30 min on ice. Cells were further labeled with secondary antibodies conjugated with the allophycocyanin (APC) fluorophore. The positive fraction of cells was gated based on the staining of the isotype control. The data were analyzed using FlowJo software.

2.5. Immunofluorescence staining

For primary pancreatic cancer tissues, paraffin-embedded, 5- μ m thick sections were deparaffinized and rehydrated, and the antigen was retrieved at 98 °C for 10 min in 10 mM citrate buffer. The tissue sections were blocked with 1% bovine serum albumin for 30 min at room temperature, and then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: Anti-vimentin and anti-E-cadherin. Secondary antibody staining was performed the next day using anti-mouse or anti-rabbit Alexa Fluor-488 and Alexa-Fluor 555, respectively. Slides were then mounted using Vectashield Mounting Medium with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) and a glass coverslip.

2.6. Western blotting analysis

Harvested cells were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer with a protease inhibitor cocktail (Roche Molecular Systems Inc., Branchburg, NJ, USA). The total protein concentration was measured using a bicinchoninic acid assay. A total of 20 μ g of the protein lysates were denatured, and separated using 10% Bis-Tris gels. The following primary antibodies were used: Anti-rabbit vimentin, anti-rabbit E-cadherin, anti-rabbit N-cadherin, and anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7. Transwell migration assay

PANC-1 cells were first labeled with anti-vimentin antibodies and then mouse IgG-binding magnetic-beads (Miltenyi Biotec) were added to the mixture. Vimentin⁺ and vimentin⁻ PANC-1 cells were then separated using a magnetic column (Miltenyi Biotec) according to the manufacturer's instructions. A Transwell migration assay was conducted using 24-well Transwell chambers with 8- μ m pores on the membrane (Corning Inc., Corning, NY, USA). Briefly, PANC-1 cells in 200 μ L of serum-free medium were loaded into the upper chamber. Then, 500 μ L of medium supplemented with 10% fetal bovine serum was added into the lower chamber. After 24 h, the cells on the underside of the membrane were stained, and cells in five random high-power fields were counted under a microscope.

2.8. Statistical analysis

Data are presented as the means \pm the standard deviation (SD). A paired or unpaired Student *t*-test was used for continuous variables, as appropriate. The *chi*-squared test was adopted for the comparison of categorical parameters. One-way analysis of variance (ANOVA) was performed to calculate the differences between multiple groups.

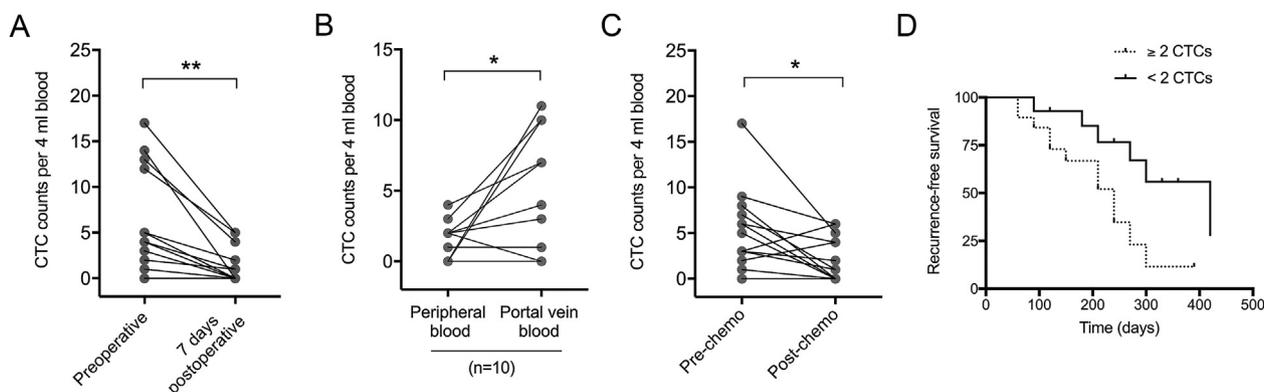


Fig. 3. Vimentin⁺ circulating tumor cells (CTCs) reveal the tumor burden and chemotherapy response. Comparison of vimentin⁺ CTC counts in blood samples from patients pre- and post-surgery (A), pre- and post-chemotherapy (B), and in peripheral and portal vein blood samples (C). Recurrence-free survival analysis based on the number of vimentin⁺ CTCs (D). **p* < 0.05, ***p* < 0.01.

Table 2
Dynamics of vimentin⁺ CTC counts and CA19-9 level in patients receiving chemotherapy.

Patient No.	Chemotherapy regimen	CTC counts		CA19-9 (U/ml)		Therapy response
		Before therapy	After therapy	Before therapy	After therapy	
1	mFOLFIRINOX	9	6	12000	240.0	PR
2	mFOLFIRINOX	7	1	133.5	2.5	PR
3	mFOLFIRINOX	6	4	347.3	88.2	PR
4	mFOLFIRINOX	1	0	11562.5	503.0	SD
5	mFOLFIRINOX	6	0	218.7	55.2	SD
6	mFOLFIRINOX	17	5	2.0	2.0	SD
7	mFOLFIRINOX	3	6	12000	12000	SD
8	mFOLFIRINOX	3	1	12000	12000	SD
9	mFOLFIRINOX	8	0	1395.8	242.9	SD
10	mFOLFIRINOX	3	2	797.0	1824.9	SD
11	mFOLFIRINOX	0	0	288.7	1451.9	PD
12	Gem + Nab-Paclitaxel	3	2	8667.6	530.0	PR
13	Gem + Nab-Paclitaxel	5	0	3479.6	2394.3	SD
14	Gem + Nab-Paclitaxel	0	0	3871.5	1940.9	SD
15	Gem + Nab-Paclitaxel	2	4	2.0	2.0	PD

CTC, circulating tumor cells; CA19-9, carbohydrate antigen 19-9; mFOLFIRINOX, modified regimen of oxaliplatin, leucovorin, irinotecan, and fluorouracil (5-FU); Gem, Gemcitabine.

Disease-free survival analysis was performed using Kaplan–Meier method. All statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA) and Prism 6.0 (Graphpad, La Jolla, CA, USA). A two-sided *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Validation of vimentin as a surface marker for pancreatic cancer cells with a mesenchymal phenotype

Vimentin was detected in pancreatic cancer cell lines with a mesenchymal phenotype, including PANC-1 and MIA PaCa-2, but not in BxPC-3 and T3M4, which exhibit an epithelial phenotype (Fig. 1A). Upon induction of EMT by hypoxia or transforming growth factor beta (TGF- β) treatment (10 ng/ml), further upregulation of mesenchymal markers vimentin and N-cadherin in PANC-1 cells was observed (Fig. 1A). Flow cytometry analysis showed that vimentin was located on the cell membrane (Fig. 1B). Importantly, the amount of vimentin on the cell surface was consistent with that of total vimentin, suggesting it could be an indicator of EMT in pancreatic cancer cells (Fig. 1B). The anti-vimentin antibody used in flow cytometry was identical to the one used for CTC capture.

To further confirm whether vimentin⁺ pancreatic cancer cells have increased migratory capacity, a critical feature of EMT, we sorted PANC-1 cells with or without cell-surface vimentin staining and performed a Transwell assay. As expected, vimentin⁺ cells exhibited a substantially higher migratory capacity than vimentin⁻ cells (Fig. 1C, *p* < 0.001). Similarly, a wound-healing assay showed enhanced closure of the wound in the presence of vimentin⁺ PANC-1 cells compared with that in the presence of vimentin⁻ PANC-1 cells (Supplementary Fig. 1). Furthermore, we observed that vimentin expression was detected on PANC-1 cells under both permeabilized and non-permeabilized conditions, indicating its location in the cytoplasm and on the cell membrane. However, vimentin was barely detectable in non-permeabilized CAFs, despite abundant expression being observed after permeabilization (Fig. 1D). In addition, using the same anti-vimentin antibody, we detected cell membrane-located vimentin in human pancreatic cancer tissue (Fig. 1E). These data suggested that vimentin might serve as a surface marker for pancreatic cancer cells with a mesenchymal phenotype.

3.2. Detection of vimentin⁺ CTCs improves the diagnosis of pancreatic cancer

We succeeded in detecting CTCs (vimentin⁺CD45⁻Hoechst⁺ or Pan-CK⁺CD45⁻Hoechst⁺) in patients with pancreatic cancer using antibody-coated microfluidic chips (Fig. 2A). In the initial 20 cases with paired blood samples, we noticed that vimentin⁺ CTCs were more frequently detected than EpCAM⁺ CTCs (median, 3 vs. 0 cell/4 ml of blood) (Fig. 2B, *p* = 0.003). To further validate the specificity of vimentin⁺ CTCs as a biomarker for pancreatic cancer, we examined the number of vimentin⁺ CTCs in 100 patients with PDAC, together with 30 healthy individuals and 16 patients with IPMN (Fig. 2C). Overall, vimentin⁺ CTCs were detected in 76% of patients with PDAC, but only in 2 out 30 healthy donors (Fig. 2D). Besides, five patients (31.3%) with IPMN had vimentin⁺ CTCs, and 10 CTCs were identified in one case. Using a cut-off value of two cells/4 ml of blood, 65% PDAC patients were positive for vimentin⁺ CTCs, while the positivity rates for the patients with IPMN and the healthy controls were 25% and 0%, respectively (Fig. 2E). The median number of CTCs for all patients with PDAC was 3 (range: 0–23 in 4 ml of blood). Importantly, CTCs improved the diagnostic power of CA19-9. The combined analysis of CTCs and CA19-9 for PDAC diagnosis showed high accuracy, with an area under curve (AUC) of 0.968 (Fig. 2F). Thus, vimentin⁺ CTCs could be a potential diagnostic biomarker for pancreatic cancer.

3.3. Vimentin⁺ CTCs correlate with the tumor burden

We then evaluated the clinical significance of vimentin⁺ CTCs in PDAC. Clinical parameters between patients with less than two CTCs were compared with those of patients with more than two CTCs (Table 1). There were no significant differences in age, sex, and CA19-9 level between the two groups. However, patients with two or more detectable vimentin⁺ CTCs had more advanced stage disease and metastasis than those with one or no CTCs (*p* = 0.015, *p* = 0.005). In parallel, patients with metastasis had significantly more CTCs than those with localized disease (median: 5 vs. 3 CTCs/4 ml blood, *p* = 0.018) (Fig. 2G). In the 40 patients who underwent surgical resection, patients with higher numbers of vimentin⁺ CTCs were associated with poor differentiation (*p* = 0.031).

We collected peripheral blood samples before surgery and at seven days after surgery in 16 patients. Except for two patients in whom CTCs were not detected before surgery, the other 14 patients demonstrated reduced CTC counts after surgery (Fig. 3A, *p* = 0.003). In particular, we failed to detect any vimentin⁺ CTCs postoperatively in six of these

patients. In the eight patients with detectable CTCs on postoperative day 7, three of them who had a peripheral blood sample available one month after surgery had undetectable CTCs. Notably, 10 patients had concomitant portal vein blood samples taken during surgery. The paired analysis showed that the CTC counts were higher in portal vein blood than peripheral blood (median: 6 vs. 2 CTCs/4 ml blood) (Fig. 3B, $p < 0.05$).

Therefore, we further tested whether this biomarker could reflect the tumor response to chemotherapy. We collected serial blood samples before and after a course of modified FOLFIRINOX (oxaliplatin, leucovorin, irinotecan, and fluorouracil (5-FU)) or Gemcitabine plus Nab-Paclitaxel chemotherapy in 15 subjects (Table 2). In the 13 patients exhibiting a response (either tumor shrinkage or stable tumor burden), their CTC counts were all decreased or remained the same, except for one case. In contrast, the two subjects defined as having progressive disease showed no reduction in CTC counts (Fig. 3C). These data confirmed that the vimentin⁺ CTC count might indicate the tumor burden and treatment response. In addition, patients with higher numbers of vimentin⁺ CTCs had inferior recurrence-free survival after surgical resection (Fig. 3D, Log-rank test: $p = 0.02$).

4. Discussion

In the present study, we reported the detection of vimentin⁺ CTCs and its clinical relevance in pancreatic cancer. As a classic molecular marker of EMT, vimentin constitutes one of the most abundant proteins in the cytoplasm of mesenchymal tumor cells. Li and colleagues created an anti-vimentin antibody that could bind with the full-length vimentin antigen and found that vimentin was present on the tumor cell surface [24,26]. Similarly, in pancreatic cancer, we confirmed that this antibody could recognize cell-surface vimentin.

Our study supported the view that the enriched vimentin⁺ circulating cells are cancerous cells originating from pancreatic cancer. First, vimentin⁺ circulating cells in healthy individuals were rare. Second, the vimentin⁺ circulating cell count in portal vein blood was significantly higher than that in peripheral blood. This result was consistent with the theoretical distribution of tumor cells in the vasculature for PDAC, reflecting the retention of CTCs in the liver [27]. A similar pattern of CTC distribution was seen in colon and lung cancers [28–30]. Third, patients who received curative tumor resection had remarkably reduced CTC counts after surgery. The good correlation with the tumor burden strongly indicated that vimentin⁺ CTCs were derived from pancreatic cancer rather than non-specific recognition of non-cancerous cells.

The performance of CTC detection in PDAC diagnosis varies according to the different techniques applied. Generally, immunological capture methods, like CellSearch[®], provide sensitivity generally below 50% [13,14], while cell size-based enrichment techniques have a better diagnostic performance [16]. Sefrioui and colleagues determined the diagnostic value of CTCs in patients with PDAC across distinct tumor stages using a filtration method and morphological characterization [31]. The sensitivity and specificity for CTCs detection were 67% and 80%, respectively, which was concordant with other reports using similar strategies. When we used a cut-off value of two cells/4 ml of blood, we obtained a sensitivity of 65% and a specificity of 100% for diagnosis of PDAC compared with that in healthy individuals. This remains inferior to CA19-9, which has a sensitivity of 79–81% and a specificity of 82–90%. Given the high specificity of vimentin⁺ CTCs detection, further exploration of its value in discriminating pancreatic malignant tumors and benign disease is warranted. In addition, vimentin⁺ cells were detectable in a fraction of patients with pancreatic cystic tumors, among whom four cases had more than two positive cells. Both subtypes of cystic neoplasm are defined as having a pre-malignant status, and a few studies have confirmed that epithelial CTCs could be found in blood of these patients [32,33]. Importantly, using genetic engineered mouse models of pancreatic cancer, a study

demonstrated that epithelial pancreatic cells entered into bloodstream in the early stage of pancreatic intraepithelial neoplasia, and maintained a mesenchymal phenotype in circulation [20]. This was in line with the current findings that vimentin⁺ CTCs were present in patients with premalignant pancreatic cystic neoplasms. Encouragingly, the combination of CTCs and CA19-9 improved the specificity and sensitivity of PDAC diagnosis; however, this result requires further confirmation using other prospective cohorts.

There are currently no effective methods to stratify early recurrence risk or to guide neoadjuvant and adjuvant therapies in PDAC. CTCs are of particular interest as a method of liquid biopsy because they are believed to be the precursors of metastasis. Some previous studies demonstrated that CTCs levels had prognostic significance [34–36]. Poruk and colleagues reported that higher total CTC numbers, as measured using a size-based enrichment method, correlated with shortened survival in a group of patients undergoing curative resection [37]. In particular, vimentin⁺ CTCs were negatively associated with progression-free survival. Our preliminary data suggested clinical applications of vimentin⁺ CTCs. For early stage disease, the immediate reduction in the number of CTCs after resection suggested its potential in monitoring postoperative recurrence. Currently, longitudinal blood samples are routinely collected in our institute to determine whether CTCs could predict early tumor relapse. Furthermore, serial sample analysis for patients receiving chemotherapy indicated the relationship between the CTC count and the tumor response. EMT contributes to chemotherapy resistance in many types of cancer, including PDAC [38,39]. Thus, future studies should focus on the significance of mesenchymal CTCs to predict the tumor response to chemotherapy.

5. Conclusions

In the present study, we demonstrated the feasibility of using cell-surface vimentin as a candidate biomarker to identify potential mesenchymal pancreatic CTCs. Vimentin⁺ CTCs could be useful in diagnosis, monitoring the tumor burden, and to predict tumor recurrence. This strategy complements the current standard EpCAM-based CTCs detection, and warrants further validation in a larger PDAC cohort for its clinical significance.

Authors' contributions

Conception and design: XL. Bai, TB. Liang, and T. Wei. Acquisition of data: T. Wei, XY. Zhang, JX. Wang, X. Li, JY. Chen, T. Ma, GG. Li, SL. Gao, JY. Lou, RS. Que, Y. Wang, TB. Liang, and XL. Bai. Analysis and interpretation of data: T. Wei, Q. Zhang, JQ. Yang, Q. Chen, XW. Dang, L. Zheng, TB. Liang, and XL. Bai. Writing of the manuscript: T. Wei, Q. Zhang, TB. Liang, and XL. Bai. Review of the manuscript: All authors.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2019.03.009>.

References

- [1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, *Ca - Cancer J. Clin.* 65 (2015) 5–29.
- [2] L. Rahib, B.D. Smith, R. Aizenberg, A.B. Rosenzweig, J.M. Fleshman, L.M. Matrisian, Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States, *Cancer Res.* 74 (2014) 2913–2921.
- [3] C. Sperti, C. Pasquali, A. Piccoli, S. Pedrazzoli, Recurrence after resection for ductal adenocarcinoma of the pancreas, *World J. Surg.* 21 (1997) 195–200.
- [4] V.P. Groot, G. Gemenetzis, A.B. Blair, R.J. Rivero-Soto, J. Yu, A.A. Javed, et al., Defining and predicting early recurrence in 957 patients with resected pancreatic ductal adenocarcinoma, *Ann. Surg.* (2018 Mar 23), <https://doi.org/10.1097/SLA.0000000000002734> [Epub ahead of print].
- [5] G. Luo, C. Liu, M. Guo, H. Cheng, Y. Lu, K. Jin, et al., Potential biomarkers in Lewis negative patients with pancreatic cancer, *Ann. Surg.* 265 (2017) 800–805.
- [6] A. Chan, I. Prassas, A. Dimitromanolakis, R.E. Brand, S. Serra, E.P. Diamandis, I.M. Blasutig, Validation of biomarkers that complement CA19.9 in detecting early pancreatic cancer, *Clin. Cancer Res. Off. J. Am. Assoc. Canc. Res.* 20 (2014) 5787–5795.
- [7] K. Pantel, R.H. Brakenhoff, B. Brandt, Detection, clinical relevance and specific biological properties of disseminating tumour cells, *Nat. Rev. Canc.* 8 (2008) 329–340.
- [8] E.S. Antonarakis, C. Lu, B. Luber, H. Wang, Y. Chen, Y. Zhu, et al., Clinical significance of androgen receptor splice variant-7 mRNA detection in circulating tumor cells of men with metastatic castration-resistant prostate cancer treated with first- and second-line abiraterone and enzalutamide, *J. Clin. Oncol. : Off. J. Am. Soc. Clin. Oncol.* 35 (2017) 2149–2156.
- [9] M.C. Liu, P.G. Shields, R.D. Warren, P. Cohen, M. Wilkinson, Y.L. Ottaviano, et al., Circulating tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer, *J. Clin. Oncol. : Off. J. Am. Soc. Clin. Oncol.* 27 (2009) 5153–5159.
- [10] C.R. Goodman, B.L. Seagle, T.W.P. Friedl, B. Rack, K. Lato, V. Fink, et al., Association of circulating tumor cell status with benefit of radiotherapy and survival in early-stage breast cancer, *JAMA Oncol.* (2018) e180163.
- [11] S. Nagrath, R.M. Jack, V. Sahai, D.M. Simeone, Opportunities and challenges for pancreatic circulating tumor cells, *Gastroenterology* 151 (2016) 412–426.
- [12] W.J. Allard, J. Matera, M.C. Miller, M. Repollet, M.C. Connelly, C. Rao, et al., Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases, *Clin. Cancer Res. Off. J. Am. Assoc. Canc. Res.* 10 (2004) 6897–6904.
- [13] F.C. Bidard, F. Huguet, C. Louvet, L. Mineur, O. Bouche, B. Chibaudel, et al., Circulating tumor cells in locally advanced pancreatic adenocarcinoma: the ancillary CirCe 07 study to the LAP 07 trial, *Ann. Oncol. : Off. J. Eur. Soc. Med. Oncol.* 24 (2013) 2057–2061.
- [14] K. Okubo, Y. Uenosono, T. Arigami, Y. Mataka, D. Matsushita, S. Yanagita, et al., Clinical impact of circulating tumor cells and therapy response in pancreatic cancer, *Eur. J. Surg. Oncol. : J. Eur. Soc. Surg. Oncol. British Assoc. Surg. Oncol.* 43 (2017) 1050–1055.
- [15] H. Huguenschmidt, K.J. Labori, C. Brunborg, C.S. Verbeke, L.T. Seeberg, C.B. Schirmer, et al., Circulating tumor cells are an independent predictor of shorter survival in patients undergoing resection for pancreatic and periampullary adenocarcinoma, *Ann. Surg.* (2018 Sep 13), <https://doi.org/10.1097/SLA.0000000000003035> [Epub ahead of print].
- [16] L. Khoja, A. Backen, R. Sloane, L. Menasce, D. Ryder, M. Krebs, et al., A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker, *Br. J. Canc.* 106 (2012) 508–516.
- [17] B. Hotz, M. Arndt, S. Dullat, S. Bhargava, H.J. Buhr, H.G. Hotz, Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer, *Clin. Cancer Res. Off. J. Am. Assoc. Canc. Res.* 13 (2007) 4769–4776.
- [18] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, *J. Clin. Investig.* 119 (2009) 1420–1428.
- [19] A. Handra-Luca, S.M. Hong, K. Walter, C. Wolfgang, R. Hruban, M. Goggins, Tumour epithelial vimentin expression and outcome of pancreatic ductal adenocarcinomas, *Br. J. Canc.* 104 (2011) 1296–1302.
- [20] A.D. Rhim, E.T. Mirek, N.M. Aiello, A. Maitra, J.M. Bailey, F. McAllister, et al., EMT and dissemination precede pancreatic tumor formation, *Cell* 148 (2012) 349–361.
- [21] C.R. Lindsay, V. Faugeroux, S. Michiels, E. Pailler, F. Facchinetti, D. Ou, et al., A prospective examination of circulating tumor cell profiles in non-small-cell lung cancer molecular subgroups, *Ann. Oncol. : Off. J. Eur. Soc. Med. Oncol.* 28 (2017) 1523–1531.
- [22] A.J. Armstrong, M.S. Marengo, S. Oltean, G. Kemeny, R.L. Bitting, J.D. Turnbull, et al., Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers, *Mol. Canc. Res. : MCR* 9 (2011) 997–1007.
- [23] A. Lecharpentier, P. Vielh, P. Perez-Moreno, D. Planchard, J.C. Soria, F. Farace, Detection of circulating tumour cells with a hybrid (epithelial/mesenchymal) phenotype in patients with metastatic non-small cell lung cancer, *Br. J. Canc.* 105 (2011) 1338–1341.
- [24] A. Satelli, A. Mitra, Z. Brownlee, X. Xia, S. Bellister, M.J. Overman, et al., Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression, *Clin. Cancer Res. Off. J. Am. Assoc. Canc. Res.* 21 (2015) 899–906.
- [25] L.M. McShane, D.G. Altman, W. Sauerbrei, S.E. Taube, M. Gion, G.M. Clark, Reporting recommendations for tumour MARKer prognostic studies (REMARK), *Br. J. Canc.* 93 (2005) 387–391.
- [26] A. Satelli, A. Mitra, J.J. Cutrera, M. Devarie, X. Xia, D.R. Ingram, et al., Universal marker and detection tool for human sarcoma circulating tumor cells, *Cancer Res.* 74 (2014) 1645–1650.
- [27] D.V. Catenacci, C.G. Chapman, P. Xu, A. Koons, V.J. Konda, U.D. Siddiqui, et al., Acquisition of portal venous circulating tumor cells from patients with pancreaticobiliary cancers by endoscopic ultrasound, *Gastroenterology* 149 (2015) 1794–1803 e1794.
- [28] R.M. Reddy, V. Murlidhar, L. Zhao, S. Grabauskiene, Z. Zhang, N. Ramnath, et al., Pulmonary venous blood sampling significantly increases the yield of circulating tumor cells in early-stage lung cancer, *J. Thorac. Cardiovasc. Surg.* 151 (2016) 852–858.
- [29] L.R. Jiao, C. Apostolopoulos, J. Jacob, R. Szydlo, N. Johnson, N. Tsim, et al., Unique localization of circulating tumor cells in patients with hepatic metastases, *J. Clin. Oncol. : Off. J. Am. Soc. Clin. Oncol.* 27 (2009) 6160–6165.
- [30] Y. Sun, W. Guo, Y. Xu, Y.H. Shi, Z. Gong, Y. Ji, et al., Circulating tumors cells from different vascular sites exhibit spatial heterogeneity in epithelial and mesenchymal composition and distinct clinical significance in hepatocellular carcinoma, *Clin. Cancer Res. Off. J. Am. Assoc. Canc. Res.* 24 (2017) 547–559.
- [31] D. Sefrioui, F. Blanchard, E. Toure, P. Basile, L. Beaussire, C. Dolfus, et al., Diagnostic value of CA19.9, circulating tumour DNA and circulating tumour cells in patients with solid pancreatic tumours, *Br. J. Canc.* 117 (2017) 1017–1025.
- [32] A.D. Rhim, F.I. Thege, S.M. Santana, T.B. Lannin, T.N. Saha, S. Tsai, et al., Detection of circulating pancreas epithelial cells in patients with pancreatic cystic lesions, *Gastroenterology* 146 (2014) 647–651.
- [33] K.E. Poruk, V. Valero 3rd, J. He, N. Ahuja, J.L. Cameron, M.J. Weiss, et al., Circulating epithelial cells in intraductal papillary mucinous neoplasms and cystic pancreatic lesions, *Pancreas* 46 (2017) 943–947.
- [34] K.E. Effenberger, C. Schroeder, A. Hanssen, S. Wolter, C. Eulenburg, M. Tachezy, et al., Improved risk stratification by circulating tumor cell counts in pancreatic cancer, *Clin. Cancer Res. Off. J. Am. Assoc. Canc. Res.* 24 (2018) 2844–2850.
- [35] C.M. Court, J.S. Ankeny, S. Sho, P. Winograd, S. Hou, M. Song, et al., Circulating tumor cells predict occult metastatic disease and prognosis in pancreatic cancer, *Ann. Surg. Oncol.* 25 (2018) 1000–1008.
- [36] K. Tjensvoll, O. Nordgard, R. Smaaland, Circulating tumor cells in pancreatic cancer patients: methods of detection and clinical implications, *Int. J. Cancer* 134 (2014) 1–8.
- [37] K.E. Poruk, V. Valero 3rd, T. Saunders, A.L. Blackford, J.F. Griffin, J. Poling, et al., Circulating tumor cell phenotype predicts recurrence and survival in pancreatic adenocarcinoma, *Ann. Surg.* 264 (2016) 1073–1081.
- [38] T. Arumugam, V. Ramachandran, K.F. Fournier, H. Wang, L. Marquis, J.L. Abbruzzese, et al., Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer, *Cancer Res.* 69 (2009) 5820–5828.
- [39] X. Zheng, J.L. Carstens, J. Kim, M. Scheible, J. Kaye, H. Sugimoto, et al., Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer, *Nature* 527 (2015) 525–530.