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Detection of Circulating Pancreas Epithelial Cells in Patients with Pancreatic Cystic Lesions

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Abstract

Hematogenous dissemination is thought to be a late event in cancer progression. We showed recently that pancreas cells can be detected in the bloodstream before tumor formation, in a genetic model of pancreatic ductal adenocarcinoma (PDAC). To confirm these findings in humans, we used microfluidic geometrically enhanced immunocapture to detect circulating pancreas epithelial cells (CECs) in patient blood samples. We captured >3 CECs/ml in 7 of 21 (33%) of patients with cystic lesions and no clinical diagnosis of cancer (Sendai criteria negative),

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8 of 11 (73%) with PDAC, and in 0 of 19 patients without cysts or cancer (controls). These findings indicate that cancer cells are present in the circulation of patients before tumors develop, which might be used in risk assessment.

Keywords

early detection; IPMN; circulating tumor cells; pancreatic cancer

A widely-accepted paradigm in cancer biology is that epithelial cancers progress in a linear manner whereby cancer-defining properties are acquired sequentially¹. In this model, cancer cells acquire metastatic potential after large primary tumors are established. However, in pancreatic ductal adenocarcinoma (PDAC), the linear progression model cannot be reconciled with clinical observations. A number of patients undergoing pancreatectomy for chronic pancreatitis will develop disseminated PDAC although only precancerous pancreatic intraepithelial neoplasias (PanINs) and no tumors are found on histologic analysis². Additionally, in patients with small primary tumors (<2cm) who have no clinical evidence of metastatic disease, five year survival after pancreatectomy is <18% due to recurrent metastatic disease³. These data suggest that metastatic seeding may occur before the formation of large primary tumors. Moreover, we recently showed that hematogenous dissemination occurs prior to tumor formation, in a lineage-labeled genetic model of PDAC⁴ at which time the pancreas contained only PanIN. Based on the clinical characteristics of PDAC and our findings within a recapitulative mouse model, we hypothesized that bloodstream seeding of pancreas-derived epithelial cells can occur in patients with clinical evidence of only precancerous lesions of the pancreas and no detectable invasive carcinoma.

To test our hypothesis, we performed a blinded prospective pilot study of three cohorts: 1) patients with no history of cancer presenting for average-risk, age-appropriate colonoscopy screening and no adenomas detected; 2) patients with precancerous cystic lesions (intraductal papillary mucinous neoplasm (IPMN) or mucinous cystic neoplasms (MCN)) of the pancreas with no evidence of tumor or metastasis on CT or MRI, who did not qualify for surgery under Sendai criteria⁵ (including no evidence of dysplasia or cancer on FNA, if done); and 3) patients with cytology-confirmed PDAC. Peripheral blood was obtained from consented patients prior to procedure. We analyzed blood samples using geometrically enhanced differential immunocapture (GEDI), a microfluidic platform that has been shown to detect circulating tumor cells from patients with prostate cancers with high sensitivity^{6, 7}. Here, we functionalized the GEDI device using antibodies to epithelial cell adhesion molecule (EpCAM) to capture circulating epithelial cells (CECs). Captured cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei and fluorescently conjugated antibodies to CD45, a universal marker of leukocytes, and cytokeratin 19 (CK19), a marker of epithelial-derived cells or pancreas and duodenal homeobox protein-1 (Pdx-1), a pancreas-specific transcription factor. A blinded observer (BJK) enumerated CECs using two definitions, A) CD45-, DAPI+ and B) CK+, CD45-, DAPI+ using a fluorescence microscope. Definition A was confirmed retrospectively with automated cell enumeration and four-color immunofluorescence for epithelial and pancreas-specific markers (Figure S1 and S2, Supplemental Methods).

We prospectively enrolled 48 patients (Table 1). Cyst- and cancer-free patients tended to be younger compared with cystic lesion and PDAC cohorts ($p=0.003$). However, there were no differences in other demographics. Most (85%) cystic lesions were classified as side-branch IPMNs. The size of cystic lesions varied from 5 to 28mm. Patients with PDAC had a wide range of primary tumor diameter (15–91mm) and tumor stage (I to IV).

Sixteen of nineteen cancer-free controls had no CECs by either definition (Fig. 1B). When CECs were detected, there were no more than 3/ml. Seven of 9 (78%) patients with PDAC had detectable CECs, with an average of 16.2 ± 19.5 CEC/ml blood ($p<0.0001$ compared to cancer-free patients by Mann-Whitney test). 8 of 20 (40%) patients with cystic lesions of the pancreas had detectable CECs, averaging 4.5 ± 7.3 CECs/ml blood ($p=0.022$ compared to cancer-free patients), and there was a significant difference in CECs across the three groups by one-way ANOVA ($p=0.015$). Interestingly, there was no significant difference in the number of CECs detected among cyst lesion patients based on the immunofluorescence definition used (Fig. 1B; black denotes definition A, red denotes CEC analysis from different patients using definition B); that is, a similar percentage of cyst lesion patients contained CECs by either definition, and, when CECs were detected in these patients, a similar concentration was found. We found no correlation with CEC count and tumor or cyst size, cancer stage or serum CA19-9 and CEA.

To confirm the pancreas origin of CECs, we stained cells for Pdx-1, a pancreas-specific transcription factor, expressed in up to 60% of all CECs in mouse models of PDAC (Fig. 1B). Adherent and GEDI-captured primary PDAC cells also expressed nuclear Pdx-1 (21% of PI34 and 10.7% of Panc-01; Fig. 1C). However, no nuclear Pdx-1 was detected within human breast (MCF-7) or prostate (LNCaP, CWR22Rv1) cancer cells or CD45+ leukocytes (data not shown). These data suggest that Pdx-1 is a specific marker of pancreas-derived cells. In our analyses, 29% of all CECs exhibited nuclear Pdx-1 staining (Fig. 1D). These data confirm that at least a portion of all GEDI-captured epithelial cells derive from the pancreas.

In conclusion, we report for the first time that pancreas epithelial cells can enter the bloodstream in patients with cystic lesions of the pancreas prior to the clinical diagnosis of cancer. Using state-of-the-art microfluidic technology⁷ and immunofluorescence staining, we confirmed the pancreas origin of captured CECs. Thus, these findings suggest that the ability to seed the bloodstream may precede the formation of detectable tumors, supporting our findings in genetic mouse models of PDAC⁴. These data are supported by the recent finding that 24.6% of resected side-branch IPMNs that do not satisfy Sendai criteria contain regions of high-grade dysplasia or invasive carcinoma⁸. Data from our mouse model predict that these cells represent early, occult cancer cells⁴, although we do not yet have evidence to support this in humans. Studies are underway to interrogate the genomic signature of CECs from cystic lesion patients—if these cells represent the earliest forms of cancer, we predict that they would contain a complement of somatic mutations associated with PDAC. Genomic analyses of CECs represent a technical challenge which recently has been elegantly addressed using massively parallel sequencing of RNA from captured tumor cells from patients with PDAC⁹; however, cyst lesion patients contain many fewer CECs, complicating genomic analysis. Further, it is still unknown if patients with CECs are

destined to form tumors. If associated with subsequent tumor formation, CEC detection could be utilized as a biomarker for cancer risk stratification in patients at-risk for PDAC. Studies underway in this regard will prospectively follow GEDI-analyzed cystic lesion patients to determine if CEC number or genomic analysis are predictive of an eventual diagnosis of PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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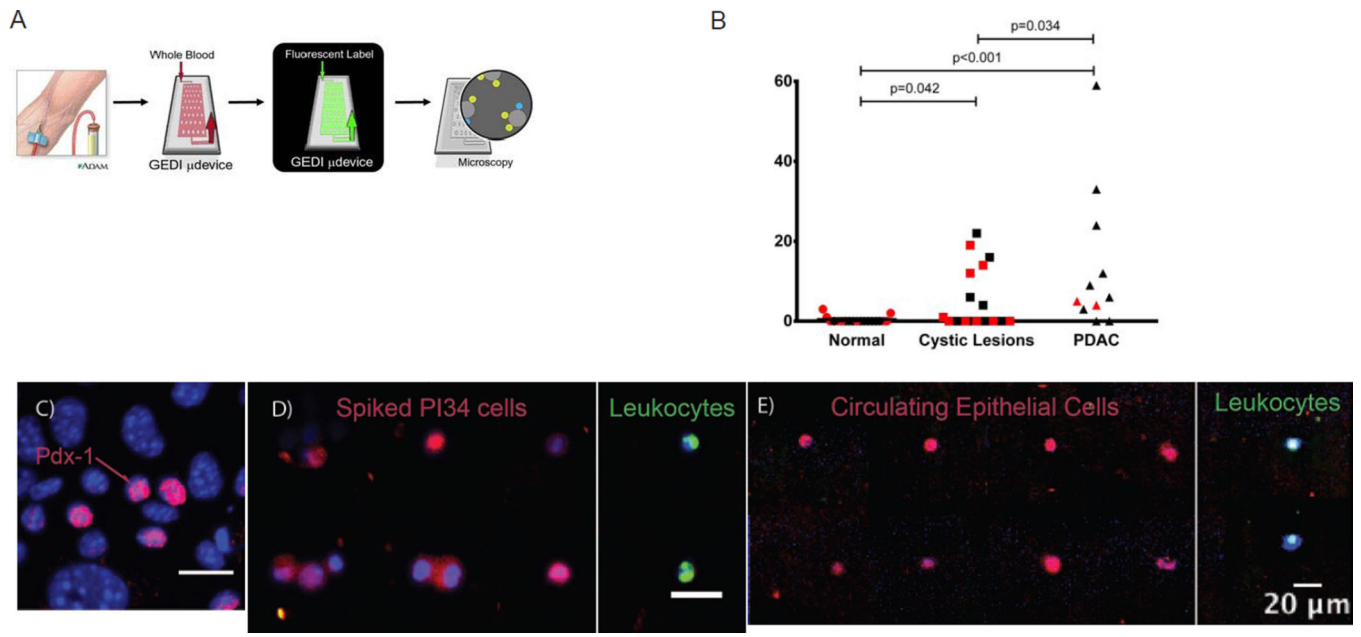


Figure 1. Detection of CECs in patients using GEDI

(A) Depiction of the GEDI device. (B) Vertical scatterplots of CEC concentrations (per ml blood) for Cancer-Free patients (Control), patients with cystic lesions of the pancreas without dysplasia or tumor (Cystic Lesion) and patients with PDAC. Lines indicate means. Bars indicate statistically significant differences by Mann-Whitney test. Representative images of individual GEDI-captured nucleated cells from (C) control human blood spiked with PI34 cells and (D) blood from a patient with PDAC. Cells were stained for CD45 (green), Pdx-1 (red) and DNA (DAPI, blue). Scale bar, 20 μ m.

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Age	Race	Sex	FHx	BMI	Smoking	E(OH) (Avg/wk)	CA19-9 (Serum)	CEA (Serum)	CECs	Size of Cyst/Tumor (mm)	Cyst type/ Cancer Stage
58	Cauc	M		20.7	Current	20			0	5	Side-branch IPMN
64	Cauc	F	Y	18.0	Never	5	46	3.8	0	20	Side-branch IPMN
73	Cauc	F		24.6	Never	0			4	14	Side-branch IPMN
69	Cauc	F		27.8	Previous	4			0 (CK)	11	Side-branch IPMN
68	Cauc	M		26.3	Previous	7			0 (CK)	3	Side-branch IPMN