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

Andrew D. Rhim,[1,2,3] Fredrik I. Thege,[4] Steven M. Santana,[5] Timothy B. Lannin,[5] Trisha N. Saha,[1,2] Shannon Tsai,[2,3] Lara R. Maggs,[2,3] Michael L. Kochman,[2,3,8] Gregory G. Ginsberg,[2,3,8] John G. Lieb,[2,3] Vinay Chandrasekhara,[2,3] Jeffrey A. Drebin,[3,6] Nuzhat Ahmad,[2] Yu–Xiao Yang,[2] Brian J. Kirby,[5,7] and Ben Z. Stanger[2,3,8]

[1]*Division of Gastroenterology, Department of Internal Medicine, Pancreatic Cancer Center, University of Michigan Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Michigan;* [2]*Gastroenterology Division, Department of Medicine,* [3]*Abramson Cancer Center,* [6]*Department of Surgery,* [8]*Department of Cell and Developmental Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania;* [4]*Department of Biomedical Engineering,* [5]*Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, New York;* [7]*Department of Medicine, Division of Hematology/Medical Oncology, Weill Cornell Medical College, New York, New York*

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Hematogenous dissemination is thought to be a late event in cancer progression. We recently showed in a genetic model of pancreatic ductal adenocarcinoma that pancreas cells can be detected in the bloodstream before tumor formation. To confirm these findings in humans, we used microfluidic geometrically enhanced differential immunocapture to detect circulating pancreas epithelial cells in patient blood samples. We captured more than 3 circulating pancreas epithelial cells/mL in 7 of 21 (33%) patients with cystic lesions and no clinical diagnosis of cancer (Sendai criteria negative), 8 of 11 (73%) with pancreatic ductal adenocarcinoma, 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 are detected, 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 sequentially.[1] 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, but no tumors, are found on histologic analysis.[2] In addition, in patients with small primary tumors (<2 cm) who have no clinical evidence of metastatic disease, 5-year survival after pancreatectomy is less than 18% owing to recurrent metastatic disease.[3] These data suggest that metastatic seeding may occur before the formation of large primary tumors. Moreover, we recently showed that hematogenous dissemination occurs before tumor formation, in a lineage-labeled genetic model of PDAC,[4] at which time the pancreas contained only pancreatic intraepithelial neoplasias. 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 3 cohorts, as follows: (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) of the pancreas with no evidence of tumor or metastasis on computerized tomography or magnetic resonance imaging, who did not qualify for surgery using the Sendai criteria[5] (including no evidence of dysplasia or cancer on fine-needle aspiration, if performed); and (3) patients with cytology-confirmed PDAC. Peripheral blood was obtained from patients who consented before the 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 to capture circulating epithelial cells (CECs). Captured cells then were stained with 4′,6-diamidino-2-

Abbreviations used in this paper: **CEC, circulating epithelial cell; DAPI, 4′,6-diamidino-2-phenylindole; GEDI, geometrically enhanced differential immunocapture; IPMN, intraductal papillary mucinous neoplasm; PDAC, pancreatic ductal adenocarcinoma; Pdx-1, pancreatic and duodenal homeobox protein-1; CK, cytokeratin 19.**

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Table 1. Patient Characteristics

	Age, y	Race	Sex	FHx	BMI	Smoking	EtOH, avg/wk	CA19-9 serum	CEA serum	CEC	Size of cyst/tumor, mm	Cyst type/ cancer stage
Cancer-free controls (n = 19)	53	Cauc	M		35.7	Never	0			0		
	40	Cauc	M		26.3	Never	1			0		
	64	Cauc	F		28.3	Never	0			0		
	61	Cauc	M		31.0	Never	1			0		
	48	Cauc	F		21.0	Never	0			0		
	62	AAM	F		35.4	Never	0			0		
	74	Cauc	F		19.8	Never	0			0		
	56	AAM	F		24.7	Never	0.5			0		
	70	Cauc	M		26.4	Previous	0			0 (CK)		
	51	AAM	F		56.6	Never	0			0 (CK)		
	60	Cauc	M		29.5	Never	0			0 (CK)		
	66	Cauc	M		21.9	Never	1.5			0 (CK)		
	84	AAM	F		29.3	Never	0			0 (CK)		
	53	AAM	F		46.1	Never	0			0 (CK)		
	59	AAM	F		26.6	Never	0			1 (CK)		
	50	AAM	F		36.6	Never	1.5			2 (CK)		
	58	AAM	F		30.2	Never	0			3 (CK)		
	73	AAM	F		26.6	Never	0			0 (CK)		
	50	Cauc	F		24.0	Never	0			0 (CK)		
Mean	**59.6**				**30.3**					**0.3 ± 0.8**		
Cystic lesion (n = 21)	67	Cauc	F		31.6	Never	0			0	9	Side-branch IPMN, multiple
	62	Cauc	F	Y	20.2	Never	3		0	0	8	Side-branch IPMN
	64	Cauc	M		34.9	Never	5		86.7	0	16	MCN
	75	Cauc	M		24.3	Never	0			0	15	Side-branch IPMN
	65	Cauc	F	Y	22.4	Never	0			6	9.5	Side-branch IPMN
	60	Cauc	M		28.3	Never	0	1.9	22	16	14	Side-branch IPMN
	72	Cauc	M		21.3	Current	3		<1	22	10	MCN
	81	Cauc	M		27.7	Never	6			0	15	Side-branch IPMN
	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
	74	Cauc	F		26.6	None	0			19 (CK)	6.5	Side-branch IPMN
	81	Cauc	M		27.5	Previous	7			1 (CK)	25	Side-branch IPMN
	58	AAM	F		32.9	None	0			14 (CK)	5	Side-branch IPMN
	74	Cauc	F		21.7	None	0			0 (CK)	25	Side-branch IPMN
	65	Cauc	F		31.4	None	0			0 (CK)	28	MCN
	79	ASAM	M		21.5	None	0			12 (CK)	23	Side-branch IPMN
	77	Cauc	F		28.5	None	0	19	1.3	0 (CK)	28	Side-branch IPMN
	80	Cauc	M		29.9	None	0			0 (CK)	25	Side-branch IPMN
Mean	**69.8**				**26.1**					**4.5 ± 7.3**		

Table 1. Continued

	Age, y	Race	Sex	FHx	BMI	Smoking	EtOH, avg/wk	CA19-9 serum	CEA serum	CEC	Size of cyst/tumor, mm	Cyst type/ cancer stage
PDAC (n = 11)	92	Cauc	M									
	65	Cauc	M		27.0	Never	0	805	8.2	0	52	Stage I
	76	Cauc	M		33.5	Never	0			12	61	Stage IV
	69	Cauc	M		29.2	Current	3	764	46	59	91	Stage II
	65	Cauc	M		26.1	Current	1	127	39	33	33	Stage IV
	59	AAM	M		37.3	Previous	0	1256		3	23	Stage IIB
	70	Cauc	F		24.8	Previous	0	912		6	15	Stage III
	73	AAM	M		19.9	Previous	0	857		9	17	Stage IV
	62	Cauc	M		22.0	Never	0	410		0	40	Stage IV
	69	Cauc	M		29.4	Current	1	862		24	16	Stage IV
	83	AAM	F		25.5	Previous	1			4 (CK)	40	Stage IV
					27.3	Never	0			5 (CK)	27	Stage IV
	Mean	**71.2**			**27.4**					**14.1 ± 18.1**		

NOTE. CK, denotes quantification of CECs using definition 2, CK+/DAPI+/CD45-. Otherwise, CECs were quantified using definition 1 (DAPI+/CD45-).
AAM, African American; ASAM, Asian American; Cauc, Caucasian; CEA, carcinoembryonic antigen; FHx, family history; MCN, mucinous cyst neoplasm.

phenylindole (DAPI) to visualize nuclei and fluorescently conjugated antibodies to CD45, a universal marker of leukocytes, and cytokeratin 19 (CK), a marker of epithelial-derived cells or pancreatic and duodenal homeobox protein-1 (Pdx-1), a pancreas-specific transcription factor. A blinded observer (B.J.K.) enumerated CECs using the following 2 definitions: (1) CD45-, DAPI+, and (2) CK+,CD45-,DAPI+ using a fluorescence microscope. Definition 1 was confirmed retrospectively with automated cell enumeration and 4-color immunofluorescence for epithelial and pancreas-specific markers (Supplementary Figures 1 and 2, Supplementary Materials and Methods).

We prospectively enrolled 48 patients (Table 1). Cyst- and cancer-free patients tended to be younger compared with the cystic lesion and PDAC cohorts ($P = .003$). 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 28 mm. Patients with PDAC had a wide range of primary tumor diameters (15–91 mm) and tumor stages (I–IV).

Sixteen of 19 cancer-free controls had no CECs by either definition (Figure 1*B*). 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 ($P < .0001$ compared with cancer-free patients by the Mann–Whitney test). Eight of 21 (40%) patients with cystic lesions of the pancreas had detectable CECs, averaging 4.5 ± 7.3 CECs/mL blood ($P = .022$ compared with cancer-free patients), and there was a significant difference in CECs across the 3 groups by 1-way analysis of variance ($P = .015$). Interestingly, there was no significant difference in the number of CECs detected among cyst lesion patients based on the immunofluorescence definition used (Figure 1*B*; black denotes definition 1, red denotes CEC analysis from different patients using definition 2); 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 carbohydrate antigen 19-9 (CA-19-9) and carcinoembryonic antigen.

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[4] (Figure 1*B*). Adherent and GEDI-captured primary PDAC cells also expressed nuclear Pdx-1 (21% of PI34 and 10.7% of Panc-01; Figure 1*C*). 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 showed nuclear Pdx-1 staining (Figure 1*D*). These data confirm that at least a portion of all GEDI-captured epithelial cells derive from the pancreas.

In conclusion, we report that pancreas epithelial cells can enter the bloodstream in patients with cystic lesions of the pancreas before the clinical diagnosis of cancer. By using state-of-the-art microfluidic technology[7] and immunofluorescence staining, we confirmed the pancreas origin of captured CECs. Thus, these findings suggest that the ability

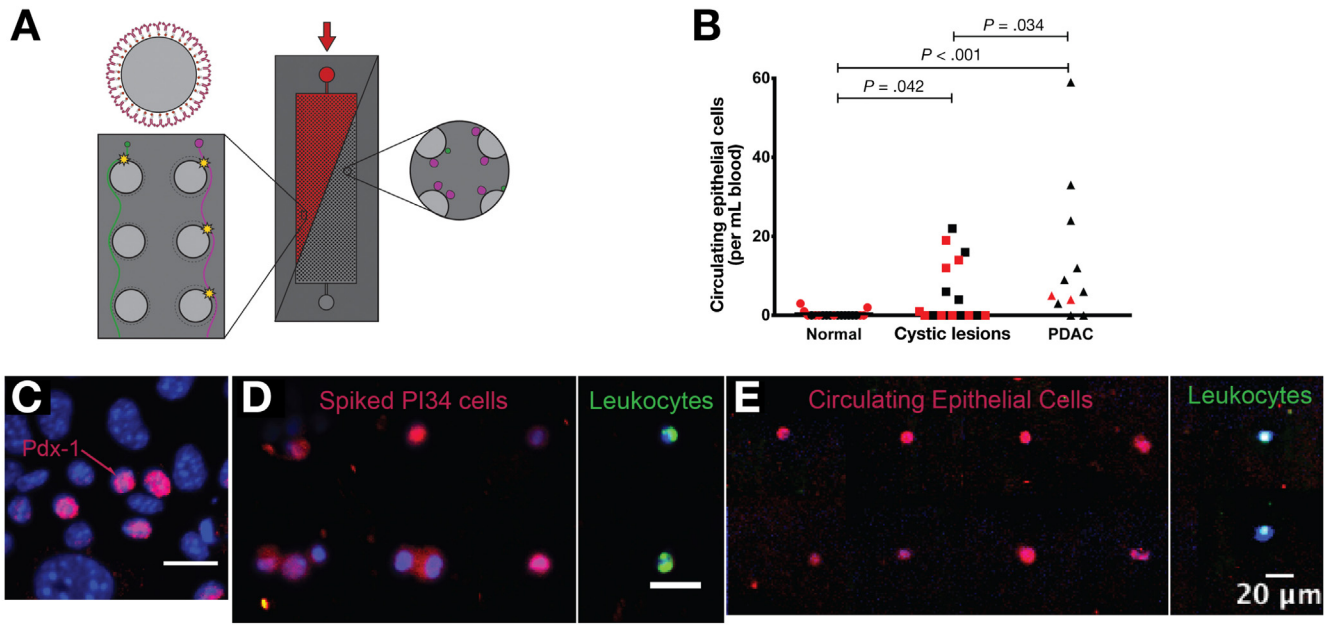

Figure 1. Detection of CECs in patients using GEDI. (*A*) Depiction of the GEDI device. (*B*) Vertical scatterplots of CEC concentrations (per milliliter blood) for cancer-free patients (control), patients with cystic lesions of the pancreas without dysplasia or tumor (cystic lesion), and patients with PDAC. *Bars* indicate statistically significant differences using the Mann–Whitney test. Representative images of individual GEDI-captured nucleated cells from (*C*) PI34 cells in culture, (*D*) control human blood spiked with PI34 cells and (*E*) blood from a patient with PDAC. Cells were stained for CD45 (green), Pdx-1 (red), and DNA (DAPI, blue). *Scale bar*: 20 μm.

to seed the bloodstream may precede the formation of detectable tumors, supporting our findings in genetic mouse models of PDAC.[4] 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 carcinoma.[8] Data from our mouse model predict that these cells represent early, occult cancer cells,[4] although we do not yet have evidence to support this in human beings. 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 that recently was addressed elegantly using massively parallel sequencing of RNA from captured tumor cells from patients with PDAC[9]; however, cyst lesion patients contain many fewer CECs, complicating genomic analysis. Furthermore, it is still unknown if patients with CECs are destined to form tumors. If associated with subsequent tumor formation, CEC detection could be used 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

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.12.007.

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Reprint requests
Address requests for reprints to: Andrew D. Rhim, MD, Division of Gastroenterology, Department of Internal Medicine, University of Michigan Medical School, 109 Zina Pitcher Place, BSRB 2061, Ann Arbor, Michigan 48108. e-mail: arhim@med.umich.edu; fax: (734) 763-2162; Brian J. Kirby, PhD, Sibley School of Mechanical and Aerospace Engineering, Cornell University, 238 Upson Hall, Ithaca, New York 14853. e-mail: kirby@cornell.edu; fax: (607) 255-1222; or Ben Z. Stanger, MD, PhD, Division of Gastroenterology, Department of Medicine, University of Pennsylvania 421 Curie Boulevard, 512 BRB II/III, Philadelphia, Pennsylvania 19104. e-mail: stanger@exchange.upenn.edu; fax: (215) 573-2486.

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Conflicts of interest
The authors disclose no conflicts.

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