# Circulating Epithelial Cells in Intraductal Papillary Mucinous Neoplasms and Cystic Pancreatic Lesions

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**Objectives:** Circulating epithelial cells (CECs) are identified in the blood of patients with intraductal papillary mucinous neoplasms (IPMNs) despite the absence of malignancy. We assessed the blood of patients undergoing resection for IPMN or other benign pancreatic lesions for CECs.

**Methods:** Peripheral blood was collected from 26 patients prior to pancreatic resection and filtered by the ISET (Isolation by Size of Epithelial Tumor Cells) method. Circulating epithelial cells were identified with antibodies to cytokeratin and Pdx1 (pancreas and duodenal homeobox protein 1), a pancreas marker.

**Results:** Nineteen patients underwent resection of an IPMN without associated malignancy. Eleven patients (58%) had cytokeratin-positive CECs. Circulating epithelial cells were significantly more likely to be found in patients with IPMNs with high-grade dysplasia ($P = 0.04$). In addition, 10 of the 11 patients with cytokeratin-positive CECs also had separate populations of cytokeratin-positive, Pdx1-positive CECs, suggesting a pancreatic source. Dual-staining CECs were more frequently found in patients with high-grade dysplasia ($P = 0.04$). Patients with IPMNs were significantly more likely to have pan-cytokeratin CECs in the blood compared with those without IPMNs ($P = 0.01$).

**Conclusions:** Circulating epithelial cells staining with potential pancreas-specific markers have been found in patients with IPMNs, even without malignancy. Circulating epithelial cells may help to differentiate patients with high-grade IPMN from lower grades of dysplasia and other pancreatic cysts.

**Key Words:** circulating epithelial cells, grade, intraductal papillary mucinous neoplasms, Pdx1

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Intraductal papillary mucinous neoplasms (IPMNs) are mucin-producing cystic lesions of the main and branch pancreatic ducts predominantly found in older individuals. While the majority of these lesions are benign, the accumulated evidence strongly supports that IPMNs are precursors to pancreatic ductal adenocarcinoma (PDAC). Several case series have demonstrated that at the time of resection anywhere from 6% to 81% of patients will be found to have an adenocarcinoma associated with their IPMN, especially those with a main-duct IPMN.[1–5] In addition, as many as 38% of patients may progress to PDAC after resection of a benign IPMN even as far out as 10 years postoperatively.[6] Certain risk factors have been identified for the progression to PDAC, thus creating specific criteria for pancreatic resection known as the Sendai Consensus Guidelines.[7] Indications for resection based on these criteria include main-duct IPMN or main-duct mixed with branch-duct IPMN, dilatation of the main duct to at least 10 mm in diameter, an associated solid component within the cyst, branch-duct IPMN greater than 3 cm in size, and individuals who are symptomatic given a higher association with high-grade dysplasia or malignancy.[7] While the goal of these guidelines is to resect lesions in high-risk patients prior to malignant progression, the criteria are neither perfectly sensitive nor perfectly specific, creating a need for further markers to identify and stratify patients for resection at highest risk of associated malignancy and dysplasia.

Circulating tumor cells (CTCs) are cells that have been shed from a tumor into the bloodstream. These circulating cells have been identified in numerous malignancies, including patients with PDAC, and are believed to be a potential marker not only for cancer but also for poor prognosis and tumor recurrence.[8] Interestingly, similar circulating epithelial cells (CECs) have been identified in patients with IPMNs despite the absence of any clinically identified malignancy. These cells have been appropriately called CECs, over CTCs, as a reflection of their likely nonmalignant source. A study of 21 patients with cystic neoplasms of the pancreas, including 18 patients with Sendai-negative IPMNs, demonstrated that 33% of patients had CECs based on expression of antibodies to epithelial cell adhesion molecule and pancreas and duodenal homeobox protein 1 (Pdx1), a pancreas-specific transcription factor.[9] However, this study was not able to confirm cyst type or the absence of pancreatic malignancy, because no patient underwent resection of the cystic lesion. A similar analysis of 21 patients with IPMNs demonstrated 13 (62%) had CECs identified based on the presence of cells with malignant-appearing morphology after Giemsa staining.[10] However, a recent study from our group suggests that identification of CECs based on morphology alone may not be accurate for the identification of these cells in PDAC.[8]

With this study, we assessed the blood of patients prior to resection of an IPMN or other cystic pancreatic neoplasm for the presence of CECs utilizing specific antibodies with comparison to patient and tumor characteristics. We demonstrate the presence of CECs in the blood of patients with IPMN but not patients with other cystic pancreatic neoplasms.

## MATERIALS AND METHODS

### Patient Selection

This study included 19 consecutive patients with IPMN, 5 patients with solid pseudopapillary neoplasms (SPN), 1 patient

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with a mucinous cystic neoplasm (MCN), and 1 patient with benign pancreatic lobular proliferation who underwent surgical resection at The Johns Hopkins Hospital between June 2013 and October 2015. These patients all consented for peripheral blood collection prior to surgical resection by written consent. Between 5 and 10 mL of venous and/or arterial blood was collected prior to surgical incision. Medical charts for all patients were reviewed, and data were collected regarding patient demographics, tumor histopathology, preoperative imaging, and perioperative factors. The pathology of all 26 patients was reviewed by a clinical pathologist and included an assessment of size, location, number, margin status, and, when applicable, grade of the lesion.

This study was performed with the approval of the institutional review board at The Johns Hopkins Hospital.

## Circulating Epithelial Cell Filtration and Identification by Immunofluorescence

Blood samples were processed using the Isolation by Size of Epithelial Tumor Cells (ISET) method (Rarecells, Paris, France), which involved mixing approximately 5 to 10 mL of patient blood with specially prepared ISET buffer and formaldehyde. This mixture was then filtered onto special membranes by the ISDT machine for further analysis. After filtration, samples were stored at −20°C until staining. Immunofluorescence was performed by rehydrating ISET membranes with 1X Tris-buffered saline prior to permeabilization with 0.2% Triton. After removal of the Triton, membranes were incubated in a 5% milk-based blocking buffer. Membranes were then incubated for 2 hours with conjugated antibodies to pan-cytokeratin (1:100, fluorescein isothiocyanate; Millipore, Danvers, Mass) and Pdx1 (1:100, Alexa Fluor 647; Abcam, Cambridge, Mass) diluted in 5% milk-based blocking buffer. Finally, membranes were washed in 1X Tris-buffered saline and affixed to glass microscope slides with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Carlsbad, Calif) before analysis by fluorescence microscope. All antibodies were appropriately tested using known positive and negative controls.

All slides were viewed under 20× magnification with the entire membrane viewed for CECs. Circulating epithelial cells were counted manually by a single user across the entire membrane, with separate counts for patients with DAPI-positive, cytokeratin-positive, Pdx1-negative CECs and patients with DAPI-positive, cytokeratin-positive, Pdx1-positive CECs. Initial exposure times were automatically identified using the Nikon NIS Elements imaging program (version 4.20.02-64 bit; Melville, NY) and corresponded to 600 milliseconds for DAPI, 1 second for cytokeratin, and 3 seconds for Pdx1. These exposure times were consistent for all 24 patients included in this study. All sections of each membrane were viewed with each separate wavelength corresponding to DAPI, cytokeratin, and Pdx1. When a candidate CEC was identified, an image under each specific wavelength was captured and saved. A CEC was defined as a cell with nuclear staining of DAPI with a diameter greater than 15 μm and cytoplasmic labeling for cytokeratin. Circulating epithelial cells were also noted for the presence or absence of Pdx1, which in the CECs identified stained predominantly in the cytoplasm. Although predominantly a nuclear transcription factor, cytoplasmic expression in pancreatic cells has been noted under certain conditions.[11–13]

## Statistical Analysis

Statistics for individual patient groups and the entire patient cohort are presented using frequencies and percentages for categorical variables and as mean values with ranges for continuous

**TABLE 1.** Comparison of Characteristics Between Patients With and Without Cytokeratin-Positive CECs

| Characteristic | All Patients (n = 19) | Pan-Cytokeratin$^+$ CTC (n = 11) | No CTC (n = 8) | *P* |
|---|---|---|---|---|
| Age, y | 66.1 (27–82) | 66.1 (27–80) | 66.1 (46–82) | 0.99 |
| Sex | | | | 0.37 |
| Male | 10 (53) | 7 (64) | 3 (37) | |
| Female | 9 (47) | 4 (36) | 5 (63) | |
| No. of IPMNs | 1.3 (1–4) | 1.4 (1–4) | 1.1 (1–2) | 0.56 |
| IPMN size, cm | 2.6 (1–9.0) | 3.0 (1.4-9.0) | 2.0 (1–4) | 0.27 |
| Grade (highest) | | | | **0.04** |
| Low | 3 (16) | 0 (0) | 3 (37) | |
| Intermediate | 12 (63) | 7 (64) | 5 (63) | |
| High | 4 (21) | 4 (36) | 0 (0) | |
| Grade (binary) | | | | 0.10 |
| Low/intermediate | 15 (79) | 7 (64) | 8 (100) | |
| High | 4 (21) | 4 (36) | 0 (0) | |
| Location | | | | 0.99 |
| Side duct | 9 (47) | 5 (45.5) | 4 (50) | |
| Main duct | 2 (11) | 1 (9) | 1 (13) | |
| Mixed | 8 (42) | 5 (45.5) | 3 (37) | |
| Margin | | | | 0.99 |
| Positive (mucin) | 5 (26) | 3 (27) | 2 (25) | |
| Negative | 14 (74) | 8 (73) | 6 (75) | |
| Associated pancreatic intraepithelial neoplasia | | | | 1.00 |
| Yes | 7 (37) | 4 (36) | 3 (37) | |
| No | 12 (63) | 7 (64) | 5 (63) | |

Data are presented as either n (%) or mean (range). Bold values are statistically significant with $P < 0.05$.

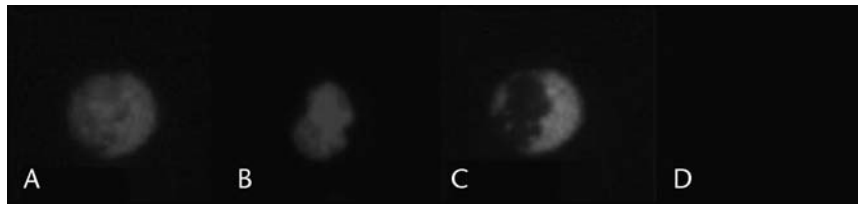


**FIGURE 1.** Circulating epithelial cells staining positive for cytokeratin. Circulating epithelial cell stains included for (A) all stains merged, (B) DAPI only, (C) pan-cytokeratin only, and (D) absence of Pdx1.

variables. Differences in patient characteristics between dichotomous cohorts were calculated utilizing Fisher's exact test. All statistical analyses were performed using STATA version 13.0 (StataCorp, College Station, Tex). Statistical significance was set at $P < 0.05$.

## RESULTS

### Assessment of CECs in Patients With IPMN

During the study period, 19 patients without a prior history of malignancy underwent pancreatic resection of an IPMN. Patient characteristics are given in Table 1. The average patient age was 66.1 years (range, 27–82 years), and 53% of patients were male. The average and median size of the IPMN were 2.6 and 2.1 cm (range, 1.0–9.0 cm), and the majority of patients (n = 15 [88%]) had only 1 IPMN by pathological analysis. The majority of patients underwent pancreaticoduodenectomy (n = 12 [63%]), followed by distal pancreatectomy with splenectomy (n = 4 [21%]), central pancreatectomy (n = 2 [11%]), or total pancreatectomy (n = 1 [5%]). More than half of all patients (n = 10 [53%]) had involvement of the main pancreatic duct. Of note, no patient with an IPMN had an associated PDAC, nor did any patient have a personal history of a concurrent or prior malignancy.

The presence of CECs was assessed by staining patient blood samples with pan-cytokeratin, an epithelial marker, and Pdx1, a pancreas specific marker. First, samples were assessed for CECs positive only for cytokeratin. Eleven patients (58%) with IPMNs were found to have CECs that stained positive for pan-cytokeratin and DAPI, a nuclear marker (Fig. 1). Differences in characteristics between patients with and without pan-cytokeratin–positive CECs are given in Table 1. There was no significant difference between any patient or tumor characteristic in patients with CECs, including IPMN size, number, or location (all $P > 0.05$). However, all patients with IPMNs with high-grade dysplasia were found to have pan-cytokeratin–positive CECs, and patients with CECs were significantly more likely to have high-grade dysplasia present at the time of surgery ($P = 0.04$).

The presence of CECs with pancreas-specific markers was then assessed by identifying CECs with both pan-cytokeratin and Pdx1. Ten patients (53%) were found to have cells that stained positive for both pan-cytokeratin and Pdx1 (Fig. 2). All 10 patients were also found to have pan-cytokeratin–positive, Pdx1-negative CECs during the same analysis, whereas 1 patient had only cytokeratin-positive CECs without any staining for Pdx1. The remaining 7 patients did not have CECs of either type. Differences in characteristics between patients with and without cytokeratin-positive, Pdx1-positive CECs are given in Table 2. Similar to the previous analysis, there was no significant difference between any patient or tumor characteristic in patients with cytokeratin-positive, Pdx1-positive CECs, including IPMN size, number, or location (all $P > 0.05$) except for grade of dysplasia. All patients with high-grade IPMNs were found to have pan-cytokeratin–positive, Pdx1-positive CECs, and patients with CECs were significantly more likely to have high-grade dysplasia present at the time of surgery ($P = 0.04$). In addition, there was a trend toward high-grade dysplasia in patients with pan-cytokeratin–positive, Pdx1-positive CECs compared with low- or moderate-grade dysplasia ($P = 0.09$).

### CECs in Non-IPMN Patients

Seven patients who underwent pancreatic resection for non-IPMN cystic disease were assessed for the presence of pan-cytokeratin CECs. This included 5 patients with SPNs, 1 patient with an MCN, and 1 patient with concern for IPMN who was found at resection to have benign lobular proliferation. Average and median ages for these patients were 56.4 and 63 years, respectively (range, 20–77 years), and 43% of patients were male. Average and median sizes of the cystic lesions were 5.1 and 3.5 cm, respectively (range, 1–17 cm). None of the 7 patients were found to have CECs as assessed by the presence of pan-cytokeratin staining. A comparison of CECs demonstrated that patients with IPMNs were significantly more likely to have pan-cytokeratin–positive CECs in the blood compared with patients without IPMNs ($P = 0.01$). The sensitivity and specificity for diagnosis were 58% and 100%, respectively.

## DISCUSSION

The identification of CECs in patients with malignancy has become an exciting potential mechanism for early detection, treatment stratification, and prediction of prognosis. However, the detection of these cells in the blood of patients without a documented cancer has led to uncertainty about the origin and significance of these cells. Multiple patient series have identified the

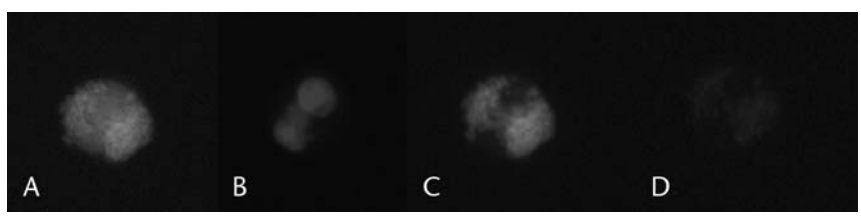


**FIGURE 2.** Circulating epithelial cell staining positive for cytokeratin and Pdx1. Circulating epithelial cell stains included for (A) all stains merged, (B) DAPI only, (C) pan-cytokeratin only, and (D) Pdx1 only.

**TABLE 2.** Comparison of Characteristics Between Patients With and Without Cytokeratin-Positive, Pdx1-Positive CECs

| Characteristic | Pan-Cytokeratin$^+$/Pdx1$^+$ CTC (n = 10) | No Pan-Cytokeratin$^+$/Pdx1$^+$ CTC (n = 9) | *P* |
|---|---|---|---|
| Age, y | 66.2 (27–80) | 66.0 (46–82) | 0.97 |
| Sex | | | 0.66 |
| Male | 6 (60) | 4 (44) | |
| Female | 4 (40) | 5 (56) | |
| No. of IPMNs | 1.3 (1–4) | 1.13 (1–2) | 0.89 |
| IPMN size, cm | 3.1 (1.4-9) | 2.0 (1–4) | 0.21 |
| Grade (highest) | | | **0.04** |
| Low | 0 (0) | 3 (33) | |
| Intermediate | 6 (60) | 6 (67) | |
| High | 4 (40) | 0 (0) | |
| Grade (binary) | | | 0.09 |
| Low/intermediate | 6 (60) | 9 (100) | |
| High | 4 (40) | 0 (0) | |
| Location | | | 0.81 |
| Side duct | 4 (40) | 5 (56) | |
| Main duct | 1 (10) | 1 (11) | |
| Mixed | 5 (50) | 3 (33) | |
| Margin | | | 0.99 |
| Positive (mucin) | 3 (30) | 2 (22) | |
| Negative | 7 (70) | 7 (78) | |
| Associated pancreatic intraepithelial neoplasia | | | 0.65 |
| Yes | 3 (30) | 4 (44) | |
| No | 7 (70) | 5 (56) | |

Data are presented as either n (%) or mean (range). Bold values are statistically significant with $P < 0.05$.

presence of epithelial or malignant-appearing cells in the blood of patients with IPMN, but correlation to underlying histopathology has been lacking. This study adds to the literature by further demonstrating the presence of CECs in patients whose absence of malignancy was confirmed by histopathologic review. In addition, our study used a unique method to identify these cells (filtering by size followed by immunofluorescence for epithelial and pancreatic markers), demonstrating that the identification of these cells is not an artifact of a single isolation method. As with 2 prior studies, we identified the presence of CECs through cytokeratin staining in more than half of all patients undergoing resection for an IPMN. Furthermore, none of these patients had any history of malignancy, and complete pathological examination of the resected pancreata did not reveal any malignancy.

Almost all of these patients had CECs that also stained for Pdx1, a marker thought to be specific to the pancreas. However, the Pdx1 staining we observed was cytoplasmic, not nuclear. Previous reports of Pdx1 staining in pancreatic neoplasms have focused on nuclear staining, but cytoplasmic staining has been reported in endocrine cells under specific conditions.[11–14] Although Pdx1 expression is suggestive of a pancreatic source, the precise implications of cytoplasmic staining in this particular clinical situation are not known. Still, identification of driver gene mutations in these CECs will be needed to definitively confirm a neoplastic pancreatic source beyond simple staining with Pdx1.

Unlike prior studies, this study is the first to compare the presence or absence of CECs with patient and tumor characteristics. Overall, there were no differences in size, number, and location of IPMN between patients with or without CECs. Interestingly, all patients with high-grade dysplasia were found to have CECs staining with pan-cytokeratin, Pdx1, or both. In addition, patients with CECs were significantly more likely to have high- or moderate-grade dysplasia compared with patients without CECs. This creates the potential use for CECs to differentiate the degree of underlying dysplasia prior to resection of an IPMN. Further study in a larger number of patients, including those with IPMN-associated adenocarcinoma, is necessary to determine and to further evaluate this potential relationship and identify if CECs are a potential marker for high-grade dysplasia.

This study also assessed the presence of CECs in a small population of patients undergoing pancreatic resection for a non-IPMN cystic neoplasm. Many of these patients underwent resection with a high suspicion for an IPMN, only to be discovered to have a different neoplasm such as SPN or MCN or, in 1 patient's case, benign proliferation of lobular pancreatic tissue. While the diagnosis of an IPMN or other neoplasm is often straightforward, in some cases imaging cannot clearly determine the etiology of the lesion. In this study, patients with IPMNs were significantly more likely to have CECs compared with patients without an IPMN. Unlike prior studies, however, no CECs were discovered in patients with non-IPMN cystic neoplasms. This may be related to the small number of patient included in this cohort, as prior research has demonstrated the presence of CECs in patients with MCN.[9] It may be possible that a larger analysis of patients with SPN and MCN would demonstrate individuals with CECs present. Further study in a larger cohort of patients with and without IPMN is needed to determine if CECs have utility as a diagnostic marker for IPMN.

In conclusion, this study demonstrates the presence of CECs in the blood of patients with IPMN without associated adenocarcinoma, expanding on prior studies by correlating the presence of these cells to underlying histopathology. In addition, this study identified CECs staining positive for Pdx1, a pancreatic marker, further suggesting a pancreatic origin. Additional study in a larger

population of patients is needed to determine the effectiveness of CECs for the diagnosis of high-grade dysplasia prior to resection. Circulating epithelial cells may have utility as a diagnostic marker to differentiate IPMNs from other cystic lesions of the pancreas, but prospective assessment in a larger patient cohort is necessary. The presence of circulating cells in patients without cancer is a provocative discovery and creates a potential mechanism to broaden our understanding of the malignant progression.

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