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CLINICAL—PANCREAS

Acquisition of Portal Venous Circulating Tumor Cells From Patients With Pancreaticobiliary Cancers by Endoscopic Ultrasound

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See Covering the Cover synopsis on page 1647.

BACKGROUND & AIMS: Tumor cells circulate in low numbers in peripheral blood; their detection is used predominantly in metastatic disease. We evaluated the feasibility and safety of sampling portal venous blood via endoscopic ultrasound (EUS) to count portal venous circulating tumor cells (CTCs), compared with paired peripheral CTCs, in patients with pancreaticobiliary cancers (PBCs). **METHODS:** In a single-center cohort study, we evaluated 18 patients with suspected PBCs. Under EUS guidance, a 19-gauge EUS fine needle was advanced transhepatically into the portal vein and as many as four 7.5-mL aliquots of blood were aspirated. Paired peripheral blood samples were obtained. Epithelial-derived CTCs were sorted magnetically based on expression of epithelial cell adhesion molecules; only those with a proper morphology and found to be CD45 negative and positive for cytokeratins 8, 18, and/or 19 and 4′,6-diamidino-2-phenylindole were considered to be CTCs. For 5 samples, CTCs also were isolated by flow cytometry and based on CD45 depletion. ImageStream was used to determine the relative protein levels of P16, SMAD4, and P53. DNA was extracted from CTCs for sequencing of select *KRAS* codons. **RESULTS:** There were no complications from portal vein blood acquisition. We detected CTCs in portal vein samples from all 18 patients (100%) vs peripheral blood samples from only 4 patients (22.2%). Patients with confirmed PBCs had a mean of 118.4 ± 36.8 CTCs/7.5 mL portal vein blood, compared with a mean of 0.8 ± 0.4 CTCs/7.5 mL peripheral blood ($P < .01$). The 9 patients with nonmetastatic, resectable, or borderline-resectable PBCs had a mean of 83.2 CTCs/7.5 mL portal vein blood (median, 62.0 CTCs/7.5 mL portal vein blood). In a selected patient, portal vein CTCs were found to carry the same mutations as those detected in a metastatic lymph node and expressed similar levels of P16, SMAD4, and P53 proteins. **CONCLUSIONS:** It is feasible and safe to collect portal venous blood from patients undergoing EUS. We identified CTCs in all portal vein blood samples from patients with PBCs, but less than 25% of peripheral blood samples. Portal vein CTCs can be used for molecular characterization of PBCs and share features of metastatic tissue. This technique might be used to study the pathogenesis and progression of PBCs, as well as a diagnostic or prognostic tool to stratify risk of cancer recurrence or developing metastases.

Keywords: Pancreatic Cancer; Endoscopic Ultrasound; EpCAM; Portal Venous Blood.

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Pancreaticobiliary cancers (PBCs), encompassing primary pancreatic and biliary tract cancers, frequently progress silently until primary tumor growth or secondary metastatic dissemination to distant organs becomes sufficient for the development of clinical symptoms. The presence of local advancement or distant metastases often precludes curative treatment and, as a result, the 5-year overall survival rate in pancreatic cancer (PC) is 7%, with biliary tract cancers only marginally better at 16% and 18%.[1] Even in the minority subset of PBC patients amenable to curative-intent resection and adjuvant chemotherapy, the 5-year overall survival prognoses remain unsatisfactory at approximately 20% to 30%[2,3] because of the development of local recurrence or distant metastases, the majority of which occur within only 2 years of resection.[4,5]

Because of the meager survival outcomes combined with a current inability to assess tumor molecular heterogeneity or perioperative risk of recurrence adequately, the clinical management of PBCs remains controversial. A prototypical example is the subset of patients categorized as having borderline resectable PC endorsed by multiple professional societies to include nonmetastatic disease with limited vascular involvement.[6] Traditionally, patients with nonmetastatic PC, including borderline resectable patients, are assessed for surgical candidacy with preoperative

***Authors share co-first authorship.**

Abbreviations used in this paper: **CTC, circulating tumor cell; CK, cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole; EpCAM, epithelial cell adhesion molecule; EUS, endoscopic ultrasound; FNA, fine-needle aspirate; IHC, immunohistochemistry; PBC, pancreaticobiliary cancer; PC, pancreatic cancer; PDAC, pancreatic ductal adenocarcinoma.**

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cross-sectional imaging and, increasingly, endoscopic ultrasound (EUS). Consensus standard treatment has evolved into surgical resection with adjuvant chemotherapy; however, given the decreased likelihood of complete (R0), margin-negative resection and poor outcomes, there has been increased consideration for neo-adjuvant therapy. Neo-adjuvant therapy theoretically may increase the ability to achieve R0 resection, however, not only is there a lack of evidence from prospective or randomized trials showing benefits, but also no clear method for defining a successful response to neo-adjuvant therapy because several reports have indicated that standard cross-sectional radiologic imaging remains insufficient.[7–9]

In PBCs, there is still a significant clinical need to determine individualized tumor molecular profiles and personalized risk for recurrence, which may allow the following: (1) better risk stratification for postoperative recurrence, (2) identification of patients benefiting from aggressive neo-adjuvant therapy, (3) sparing low-risk patients from potentially unnecessary adjuvant therapy, and/or (4) sparing high-risk patients from undergoing invasive surgery and the associated morbidity and mortality risk that ultimately would not improve survival.

Circulating tumor cells (CTCs) shed into the blood from primary tumors hold promise in serving as an informative biomarker to address this clinical need. CTCs have been explored as a minimally invasive tool for assessing solid-tumor burden, with subsequent analyses validating CTC enumeration as a predictive and prognostic indicator of disease-free progression and overall survival.[10] However, peripheral CTCs are extremely rare with estimates of 1 tumor cell per 1 billion circulating blood cells.[11] This low value has been attributed in part to CTC biophysical factors, including platelet adhesion and circulating in clusters, irregular shapes, and at sizes larger than capillaries, which in certain malignancies can result in hepatic filtration during portal circulation transit.[12,13] The concerns of hepatic filtration, further supported by location patterns of PC tumor metastases, support the consideration of a differential CTC yield by blood collection site.

This study evaluated the feasibility and safety of sampling portal venous blood via EUS to enumerate epithelial cell adhesion molecule (EpCAM)-positive (+) portal venous CTCs.[14] We compared the number of CTCs obtained from the portal vein with peripheral blood in patients with non-metastatic and metastatic PBCs and assessed the capability of completing genomic and proteomic profiling of flow-sorted, isolated CTC fractions from EUS-obtained portal venous blood.

Materials and Methods

Patient Selection

Eighteen patients referred for EUS with cross-sectional imaging and clinical symptoms consistent with PBCs were enrolled prospectively with written consent in an Institutional Review Board–approved study at the University of Chicago Medicine.

Portal Vein Sampling

EUS for PBC staging and/or diagnosis confirmation with on-site cytopathologic analysis of fine-needle aspirates (FNA) was performed in standard fashion. Under EUS guidance, the left and right portal veins were identified. After verifying flow signal by Doppler, a 19-gauge EUS-FNA needle was advanced transhepatically into the portal vein and 2–4 aliquots of 8.5 mL blood were aspirated (Figure 1) and placed in CPT tubes (catalog number: 02-685-125, Vacutainer Glass Mononuclear Cell Preparation Tubes; BD, Franklin Lakes, NJ) and CellSave Preservative Tubes (cat: 7900005; Janssen Diagnostics, LLC, Raritan, NJ). The puncture site was monitored under EUS for complications. Routine peripheral blood samples were obtained before EUS in parallel and processed identically.

CTC Enumeration

For all patients (N = 18), portal venous blood and peripheral blood samples were analyzed using the CellSearch Kit (cat: 7900001; Janssen Diagnostics, LLC) as previously described.[15] In brief, 7.5 mL of whole peripheral blood and portal vein blood were collected in CellSave Preservative Tubes containing Na_2–EDTA and cell preservative for the epithelial cells. The buffy coat was isolated and epithelial-derived CTCs were sorted magnetically by ferromagnetic labeling with surface anti-EpCAM antibody. Isolated cells subsequently were stained for intracellular anticytokeratins 8, 18, and/or 19 phycoerythrin (PE) antibody, 4′,6-diamidino-2-phenylindole (DAPI), and anti-CD45 allophycocyanin (APC) antibody for leukocyte depletion. Only epithelial cells with consistent, cell-like morphology that were also cytokeratin 8, 18, and/or 19 +, DAPI+, and CD45-

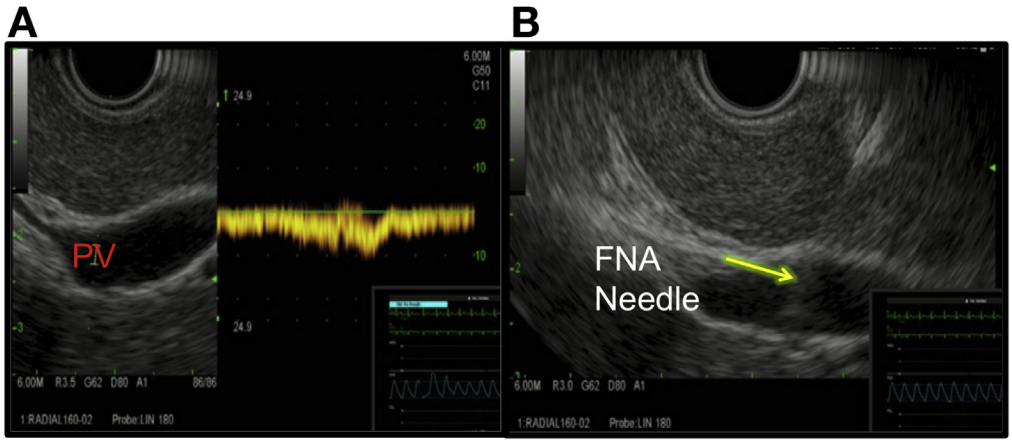


Figure 1. EUS-guided access of the portal vein. (*A*) The portal vein is identified under EUS guidance with Doppler wave verification. (*B*) EUS-guided, transhepatic, FNA puncture of the portal vein with a 19G EUS FNA needle for portal venous blood acquisition. PV, portal vein.

were counted as CTCs. As part of the standard CellSearch protocol, clusters identified by review were considered to be a single event for enumeration purposes if fewer than 5 cells were present.

Cell Sorting

In a subset of patients (n = 5), whole peripheral and portal venous blood samples were collected into CPT tubes and centrifuged for 30 minutes at 1500*g* at room temperature as previously described.[16] Briefly, the mononuclear layer cells were isolated according to the manufacturer's instructions and washed twice with phosphate-buffered saline. Cells then were stained with Alexa Fluor 488 EpCAM at 5 uL per million cells (cat: 324210; Biolegend) and CD45 antibody at 1 uL per million cells (cat: Q10156, QDot 800 CD45; Invitrogen) for 30 minutes on ice. AGS cells (human gastric cancer cell line obtained from ATCC, Manassas, VA) were prepared as an EpCAM+ control, and unstained AGS cells served as negative controls. Cell sorting was performed using standard procedure either by BD FACSARIA III or a Bio-Rad S3 cell sorter. With flow cytometry CTC isolation, CD45-, EpCAM+, and DAPI+ cells were collected as CTC cells for further genomic and proteomic analyses.

Cell Live-Imaging

In a subset of patients (n = 5), mononuclear layer cells were isolated as described earlier, washed, and depleted for CD45- cells by an AutoMACS machine (Miltenyi Biotec, San Diego, CA) after incubation with biotin anti-human CD45 (cat: 304004; Biolegend, San Diego, CA) and antibiotin microbeads (cat: 120-000-900; Miltenyi Biotec). CD45- cells were collected and fixed with 4% formaldehyde. The following day, the cells were stained with EpCAM (5 uL per million cells) and CD45 (1 uL per million cells) antibodies covered from light on ice for 30 minutes, and then washed with phosphate-buffered saline. This was followed by a cell membrane permeation step (Foxp3/Transcription Factor Staining buffer kit, cat: 00-5523-00; eBioscience, San Diego, CA), and cells were stained intracellularly for 1 hour covered from light at room temperature for p53 (4 uL per million cells, PE anti-p53, cat: 645806; Biolegend), p16 (5 uL per million cells, anti-CDKN2A/p16, Alexa Fluor 647, cat: bs-0740R-A647; Bioss, Woburn, MA), smad4 (5 uL per million cells, anti-smad4, PE Cy 5.5, cat: bs-0585R-PE-Cy5.5; Bioss), and nuclear-stained by FxCycle violet (1:10,000; cat: F10347; Invitrogen, Carlsbad, CA) for 15 minutes. AGS gastric cancer cells were used as a positive control for each antibody for compensation. Stained samples were scanned by ImageStream X (Amnis/EMD Millipore, Merck KGaA, Darmstadt, Germany), and data were analyzed by IDEAS software to provide the median absolute fluorescence intensity for each sample for each antibody conjugate. Relative expression levels were determined as the fold-change compared with the AGS cell line control after normalizing to the mean aspect ratio of the bright field.

CTC Genomic Analyses

After a whole-genome amplification step using the GenomePlex Single Cell Amp Kit (cat: WGA4; Sigma-Aldrich, St. Louis, MO), targeted Sanger sequencing was performed for *KRAS codons 12/13* and, in 1 selected patient, the *CTNNB1* region (*p.G34E* mutation), with correlation to parallel results obtained from next-generation sequencing of a metastatic lymph node biopsy sample using the Foundation One clinical assay (Foundation Medicine Inc, Cambridge, MA). Concurrent paired portal venous white blood cell–sorted DNA was used as sequencing controls for *KRAS* sequencing.

Immunohistochemistry

After deparaffinization and rehydration, tissue sections were treated with EDTA, pH 9, in a steamer. Anti-Smad4 antibody (sc-7966; Santa Cruz Biotechnology, Dallas, TX) (1:20) was applied on tissue sections for a 1-hour incubation at room temperature in a humidity chamber. After Tris-buffered saline wash, the antigen–antibody binding was detected with the Bond polymer refine detection system (DS9800; Leica Microsystems, Buffalo Grove, IL) and 3,3′-diaminobenzidine tetra hydrochloride-positive chromogen (K3468; DAKO, Carpinteria, CA). Tissue sections were immersed briefly in hematoxylin for counterstaining and were covered with cover glasses. The University of Chicago clinical immunohistochemistry (IHC) laboratory completed staining for p53 (p53 Ab-6, clone DO-1, 1:200; Thermo Scientific) and p16 (p16 [JC8], sc-56330, 1:50; Santa Cruz Biotechnology).

Statistical Methods

Results are expressed as means, medians, SDs, and ranges. Comparisons between numbers of CTCs in peripheral blood and portal venous blood were performed using paired Student *t* test. A *P* value less than .05 was considered statistically significant. Statistical analysis was performed using STATA 12.1 software (StataCorp LP, College Station, TX).

Results

Patient Characteristics, Safety, and Feasibility

The mean age of the 18 patients was 66.1 years (SD, ±10.7 y; range, 51–92 y). There were 14 men (78%) and 4 women (22%), with a racial distribution of 14 Caucasian patients (78%) and 4 African American patients (22%). Further patient characteristics, clinical staging, and sample findings are summarized (Table 1). EUS-FNA of the primary tumor confirmed adenocarcinoma in 94.4% of the patients (17 of 18 patients). Fourteen patients were confirmed to have pancreatic ductal adenocarcinoma (PDAC), 2 patients with cholangiocarcinoma, 1 patient with ampullary adenocarcinoma, and 1 patient with multiple side-branch intraductal papillary mucinous neoplasms.

In the patients with confirmed PDAC, tumor size ranged from 27 to 50 mm (mean, 35.8 mm; SD, ±7.9 mm) by EUS or cross-sectional imaging. Seven of the 14 patients with PDAC were determined to have borderline resectable disease with a clinical stage of IIB or lower; however, all 7 patients had nonceliac axis or superior mesenteric artery vascular involvement characterized by either abutment or encasement of the portal vein, hepatic artery, or splenic artery.

Patients were observed immediately under EUS guidance after portal venous blood sampling for a minimum of 5 minutes in the endoscopy suite and for 45 minutes after the procedure in the recovery area. Telephone calls were made 24 hours and 7 days after the procedure to further assess recovery. No immediate or delayed complications

Table 1. Patient and Tumor Characteristics With Associated CTC Enumeration

Diagnosis	Clinical stage	Primary tumor location	Size, *mm*	Path	Age, *y*	Race	Sex	Family history of PBC	Smoking history	Alcohol history	CA 19-9	Peripheral CTC test in 7.5 mL of blood	Portal vein CTC test in 7.5 mL of blood[a]	Chemotherapy before CTC	Distant metastases	Resectable
Pancreas mass	IIA	Body	27	AC	77	Cauc	M	No	Former	None	Increased[b]	0	101	No	No	Borderline
Pancreas mass	IIA	Body	30	AC	92	Cauc	F		Never	Active, mild	428	0	14	No	No	Borderline
Pancreas mass and IPMT	II	Head and tail	36	AC	71	Cauc	M	No	Former	None	3411	0	75	No	No	Borderline
Pancreas mass	IIB	Head	40	AC	65	Cauc	F	No	Never	Active, mild	569	0	62	No	No	Borderline
Peripancreatic mass and duodenal nodule	IIB	Head	50	AC with NET features	61	Cauc	M	No	Former	Former	55	0	265	Yes	No	Borderline
Pancreas mass	IIB	Head	43	AC	51	Cauc	M	Yes	Never	None	238	0	1 (1)	Yes	No	Borderline
Pancreas mass	IIB	Neck	34	AC	70	Cauc	F	No	Active	None	1595	1	23 (16)	No	No	Borderline
Pancreas mass	III	Body	40	AC	66	AAM	M	No	Former	Former	93	0	9	No	No	Unresectable
Pancreas mass	III	Neck	30	AC	74	Cauc	M	No	Former	Former	642	0	102	No	No	Unresectable
Pancreas mass	IV	Body	30	AC	64	Cauc	M	No	Never	Former	7896	0	132	No	Yes (to liver)	Unresectable
Pancreas mass	IV	Head and neck	50	AC	58	AAM	M	No	Never	None	15,300	0	17	No	Yes (to liver)	Unresectable
Pancreas mass	IV	Body	26	AC	57	Cauc	F	Yes	Never	None	866,900	2	429 (417)	No	Yes (to liver)	Unresectable
Pancreas mass	IV	Body	35	AC	56	AAM	M	No	Former	Active, mild	748	7	516 (379)	No	Yes (to liver)	Unresectable
Pancreas mass	IV	Head	30	AC	64	AAM	M		Former	Former	39.3	0	13	Yes	Yes (to liver)	Unresectable
Ampullary/distal CBD stenosis	IIA	Ampulla	20	AC	52	Cauc	M	No	Never	Active, mild	877	3	185	No	No	Resected
Distal CBD mass	III	Distal CBD	17	Chol	64	Cauc	M	No	Active	Former	109	0	23	No	No	Resected
Hilar bile duct mass	IV	Hilar	23	Chol	75	Cauc	M	No	Never	None	1843	0	45	No	Yes (to liver)	Unresectable
Pancreas sb-IPMNs	N/A	Head and body	80	IPMN	82	Cauc	M	No	Former	Former		0	1	No	No	

AAM, African American; AC, adenocarcinoma; NET, neuroendocrine tumor features; Cauc, Caucasian; Chol, cholangiocarcinoma; CBD, common bile duct; IPMN, intraductal papillary mucinous neoplasm; IPMT, intrapancreatic mucosal tumor; sb-IPMN, side-branch intraductal papillary mucinous neoplasm; active, mild, defined as no more than 1 drink/day for women and no more than 2 drinks per day for men.

[a]CTC values with additional value in parenthesis indicating the replicate.

[b]Number not available.

from EUS-guided portal vein sampling, including hematoma formation or gastrointestinal bleeding, were observed.

CellSearch CTC Enumeration

In all 18 patients, the mean portal venous CTCs/7.5 mL was 111.8 (SEM, ±35.3; median, 53.5) compared with 0.7 peripheral CTCs/7.5 mL (SEM, ±0.4; median, 0; $P < .01$) (Figure 2). In the 17 patients with confirmed PBCs, the mean portal venous CTCs/7.5 mL was 118.4 (SEM, ±36.8; median, 62.0; range, 1–516) compared with 0.8 peripheral CTCs/7.5 mL (SEM, ±0.4; $P < .01$). Patients with non-metastatic, resectable, or borderline resectable PBCs had a mean portal venous CTC/7.5 mL count of 83.2 (median, 62.0; range, 1–265) as compared with patients with unresectable PBCs with a mean portal venous CTC/7.5 mL count of 157.9 (median, 73.5; range, 9–156; $P = .23$). To assess the precision of CTC enumeration by CellSearch, in 4 of 14 patients a second sample of portal venous blood was aspirated and, in each patient, the enumeration was replicated with good correlation between samples (Table 1, values in parentheses). A CellSearch review of samples identified rare CTC clusters within portal venous blood (Figure 2*B*).

Pancreatic Ductal Adenocarcinoma

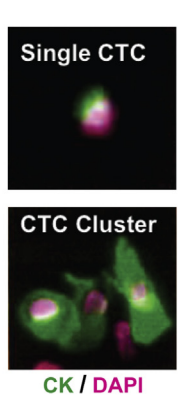
Fourteen patients were diagnosed by EUS-FNA with PC. In this cohort, all patients had CTCs detected in the portal vein, as opposed to only 3 patients with peripheral CTCs detected. The mean portal venous CTCs/7.5 mL was significantly higher at 125.64 (median, 68.5; range, 1–516), whereas the mean peripheral CTCs/7.5 mL was 0.7 (median, 0; range, 0–7; $P = .01$). In 3 patients who had received chemotherapy before portal vein sampling, all continued to have portal vein CTCs detected, ranging from 1 to 265 CTCs/7.5 mL.

Biliary Tract Cancers

Two patients, 1 patient with stage III (resectable) and the other patient with stage IV (unresectable) cholangiocarcinoma, had a CTC enumeration of 23 CTCs/7.5 mL

A

	Number of samples	Range of CTCs/7.5mL of peripheral blood				Range of CTCs/7.5mL of portal venous blood				Samples with >5 CTCs/7.5mL peripheral blood (%)	Samples with >5 CTCs/7.5mL portal venous blood (%)
		0-5	5-25	25-100	>100	0-5	5-25	25-100	>100		
		Number of samples (%)				Number of samples (%)					
Pancreatic cancer	14	13 (92.9)	1 (7.1)	0	0	1 (7.1)	5 (35.6)	2 (14.3)	6 (42.9)	7.1	92.9
Biliary cancers	3	3 (100)	0	0	0	0	1 (33.3)	1 (33.3)	1 (33.3)	0	100
Non-neoplastic IPMN	1	1 (100)	0	0	0	1 (100)	0	0	0	0	0



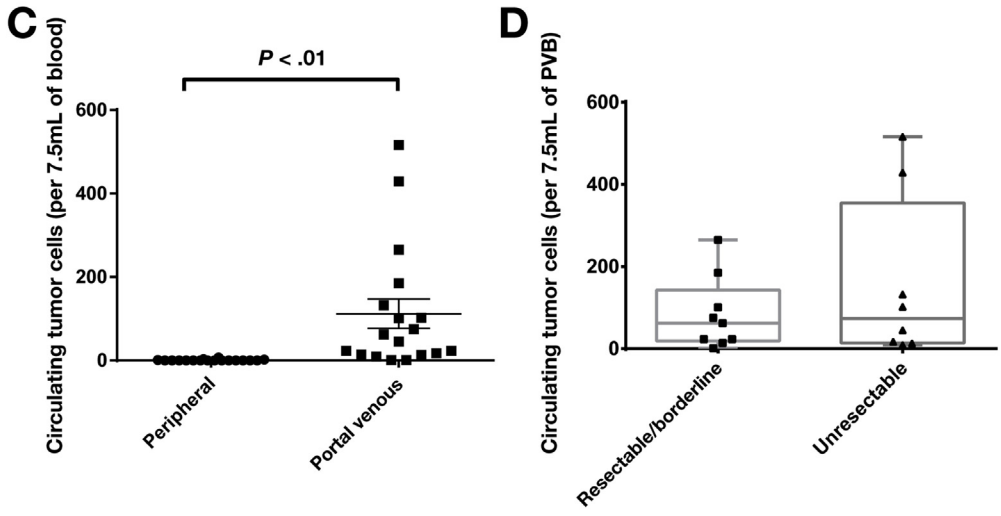


Figure 2. CTC enumeration from peripheral blood and portal venous blood. (*A*) Summary table of CTC enumeration per 7.5 mL of peripheral and portal venous blood by malignancy type. (*B*) Representative immunofluorescence staining of a single CTC and a CTC cluster captured by CellSearch enumeration (DAPI, magenta; CK, green) (*C*) Vertical scatter plot with the means ± SEM for CTCs enumerated from the peripheral blood and portal venous blood in patients with confirmed PBCs. *Bar* indicates a statistically significant difference using paired *t* test. (*D*) *Box* and *whisker plot* with individual points for CTC enumeration per 7.5 mL of portal venous blood (PVB) in all patients with confirmed PBCs and the subset of patients with borderline-resectable or resected PBCs vs unresectable PBCs. IPMN, intraductal papillary mucinous neoplasm.

and 45 CTCs/7.5 mL of portal venous blood, respectively, although both patients had a complete absence of peripheral CTCs. A patient with stage IIA ampullary adenocarcinoma (invasion of duodenal wall and pancreas) had 185 CTCs/7.5 mL of portal venous blood compared with a peripheral CTC count of 3 CTCs/7.5 mL.

Nonmalignant Intraductal Papillary Mucinous Neoplasm

A single patient with multiseptated cystic lesions consistent with side-branch intraductal papillary mucinous neoplasms was assessed as a nonmalignant cystic lesion control and was found to have 1 detected CTC in portal venous blood.

Exploratory Translational Correlative Studies

Expression loss of 1 or more tumor suppressors in PBCs, including *TP53*, *SMAD4*, and *p16/CDKN2A*, has been correlated with a worse prognosis.[17] To evaluate the ability to determine relative expression levels of these proteins in the isolated EpCAM+, DAPI+ CTCs from CD45-depleted portal venous blood (Figure 3*A*), we included immunofluorescent staining of these 3 proteins during the EpCAM flow cytometry via ImageStream technology and compared the relative signal intensity with AGS control cell lines (Figure 3*B*) with known high/low expression of each respective protein. EpCAM+ flow identified individual CTCs (Figure 3*C*) and CTC clusters (Figure 3*D*). The CTCs showed a relative gain of p53 (1.3-fold higher than controls), and smad4 protein expression (1.6-fold higher than controls), with reduced p16 expression (0.45-fold) relative to the AGS control cells (Figure 3*E*). Immunohistochemistry of a metastatic lymph node from this patient confirmed the loss of p16 expression, positive expression of smad4, and heterogeneous levels of p53 expression (Figure 3*F*). The CTC immunofluorescence patterns of these proteins also were compared with IHC of AGS cells with relatively similar patterns of expression. Further representative portal venous CTCs in an additional 4 patients are shown in Supplementary Figure 1.

Genomic sequencing of DNA extracted from the same metastatic lymph node biopsy as described earlier (Foundation One) showed the *MAP2K1*:NM_002755:c.167A>C_p.Q56P (0.13,1125, *PIK3CA*:NM 006218:c.331A>G p.K111E [0.13,765]), and CTNNB1:NM_001904:c.101G>A_p.G34E (0.06,851) mutational profile. *TP53*, *SMAD4*, *p16/CDKN2A*, and commonly mutated *KRAS* all were wild type. To confirm that the EpCAM+ portal venous CTCs indeed were derived from the pancreatic tumor, we performed targeted sequencing of the *CTNNB1* region (*p.G34E* mutation) within the whole-genome–amplified portal venous CTC-derived DNA. The mutation also was identified in this portal venous blood–derived sample (Figure 4). *KRAS codons 12/13* sequencing, which also was tested given its high frequency of mutation in PBCs, was confirmed as wild type and was consistent with the primary tumor profile. In 3 additional patients, *KRAS codons 12/13* sequencing of whole-genome–amplified portal venous CTC-derived DNA identified the presence of Gly13Asp (GGC>GAC) mutations supportive of derivation from primary malignancy (Supplementary Figure 2).

Discussion

The results from this study show that minimally invasive EUS-guided acquisition of portal venous blood for CTC enumeration and isolation is feasible and safe in PBCs and, more importantly, is capable of identifying CTCs in portal venous blood (mean, 83.2 CTCs/7.5 mL; median, 62 CTCs/7.5 mL), with relative paucity in peripheral blood (mean, 0.4 CTCs/7.5 mL; median, 0 CTCs/7.5 mL) in nonmetastatic borderline resectable PBCs. We also confirmed that isolation of CTCs from portal venous blood provides a sufficient quantity of cells to perform genomic and proteomic tumor profiling successfully.

CTCs enumerated from the peripheral blood have been shown to be informative biomarkers for progression-free survival and overall survival in patients with metastatic breast,[10] prostate,[18] and colon cancers.[19] Relative to these other epithelial malignancies, the CellSearch EpCAM-positive–dependent system consistently has identified low numbers of peripheral blood CTCs in PC.[20] Regardless of methodology, whether by antibody-mediated immunocapture of epithelial surface markers (EpCAM/MUC1), microfluidic selection,[11] or size-based filtration,[20] the ability to detect peripheral blood CTCs have been limited to metastatic PC. Initial research into the ability to detect PC CTCs has focused predominantly on metastatic disease. A preliminary study of 16 patients with metastatic PC identified a mean of 2 CTCs/7.5 mL of peripheral blood.[15] Kurihara et al[21] subsequently evaluated 26 patients with PC, of whom 25 had stage IV disease and 1 had stage III disease. In this cohort, the stage III patient had no detected peripheral blood CTCs and only 11 of 25 of the stage IV patients had measurable CTCs. In the 11 patients with peripheral blood CTCs, the median was 5 CTCs/7.5 mL (range, 1–105 CTCs/7.5 mL; mean, 17 CTCs/7.5 mL). Negin et al[22] studied 40 patients with metastatic PC and found 50% of patients to have detectable peripheral blood CTCs, with a median of fewer than 0.5 CTCs/7.5 mL (range, 0–20 CTCs/7.5 mL). Recently, the CellSearch EpCAM+ system was compared with a size-based, surface marker–independent filtration method (isolation by the size of the epithelial tumor cells [ISET]) in 31 patients with metastatic or inoperable PC.[20] Similarly, the CellSearch system identified a median number of 0 CTCs/7.5 mL (range, 0–144 CTCs/7.5 mL) and the size-based filtration method identified a slightly higher number with median of 9 CTCs/7.5 mL (range, 0–240 CTCs/7.5 mL).

The lack of efficient CTC isolation in peripheral blood has resulted in increased interest in alternative platforms including microfluidic capture. A recent report identified circulating epithelial cells using an immunocapture, microfluidic capture method in pancreatic cystic lesions and in 11 patients with PC.[23] In the PC cohort, 7 patients had stage IV disease, with the remaining 4 patients diagnosed with stages I–III disease. In 3 of 4 patients with nonmetastatic disease, circulating, nonleukocyte, nucleated cells were

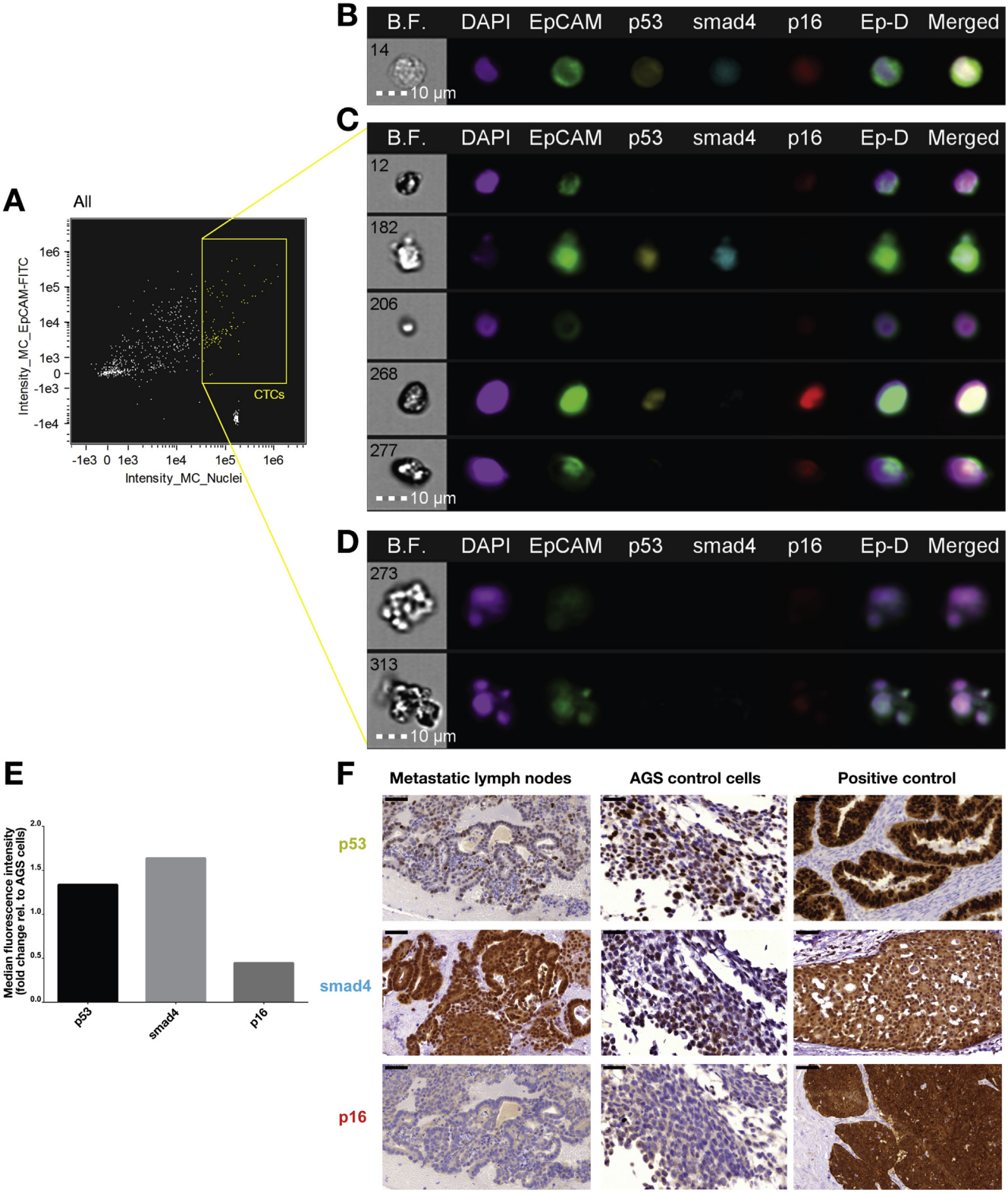


Figure 3. Tumor-suppressor genes, *p16/CDKN2A*, *TP53*, and *SMAD4* protein expression in AGS control cells and portal venous CTCs. (*A*) Flow cytometry of CD45-depleted portal venous blood gated by ImageStream for EpCAM+, DAPI+ CTCs. Representative immunofluorescent staining of the proteins in (*B*) individual AGS control cells and (*C*) portal venous CTCs during EpCAM+ flow cytometry via ImageStream technology. (*D*) Representative immunofluorescent staining of the proteins in CTC clusters identified in portal venous blood. (*E*) Median intensity for immunofluorescence of CTC p53, smad4, p16 proteins relative to AGS control cell line normalized to mean cell size (mean aspect ratio of the bright field). (*F*) Immunohistochemistry confirmation of positive smad4, loss of p16, and heterogeneous p53 expression in a metastatic lymph node relative to AGS control cells and positive control tissues (40× magnification; *scale bars*: 50 μm).

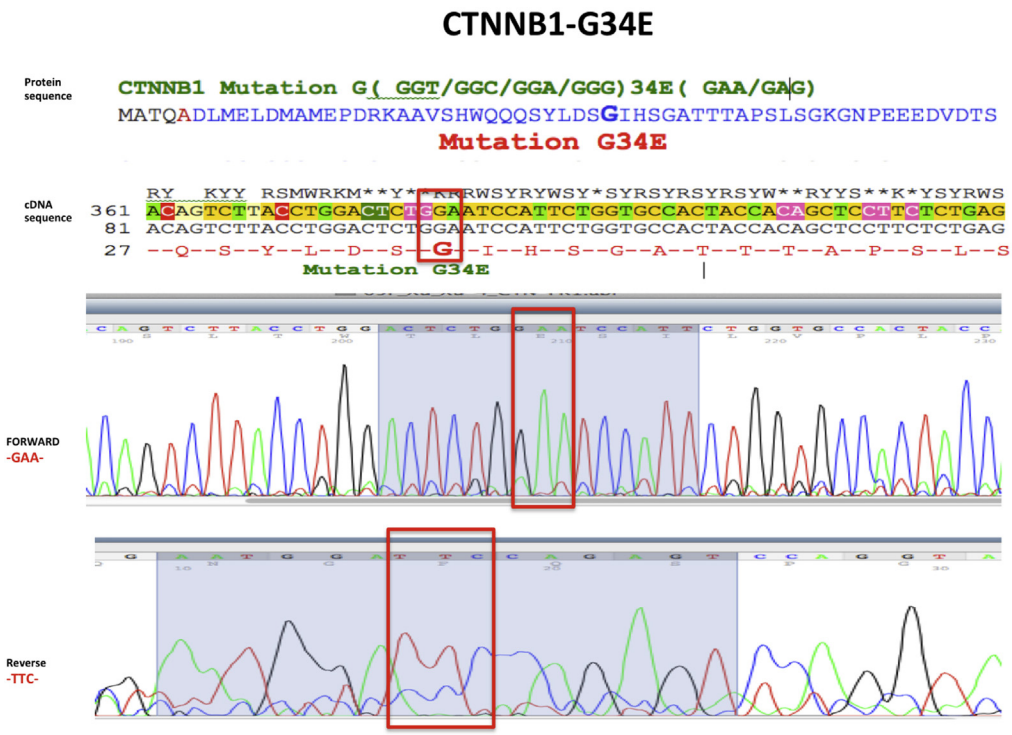


Figure 4. Confirmation of *CTNNB1* mutation detected in CTC DNA. Top 2 sequences show reference protein (*top row*) and complementary DNA (cDNA) (*second row*) sequences as G (glycine) and GGA, respectively. Bottom 2 sequencing traces show forward GAA (*third row*) and reverse TTC (*bottom row*) sequencing of portal venous CTCs isolated from the patient consistent with G34E missense mutation as found in a metastatic lymph node. *Red boxes* highlight the sequencing location of the *CTNNB1* gene with G>A nucleotide change resulting in a G34E mutation.

identified in a range of 3–59 per mL of peripheral blood. The discrepancy in enumeration relative to our investigation likely is owing in part to the sensitive, but nonspecific, methodology in defining a circulating epithelial or tumor cell. Rhim et al[23] used a definition of CD45-, DAPI+, however, when a more stringent definition was used, namely CK+, CD45-, DAPI+, the numbers of cells in the 2 reported stage IV pancreatic cancer patients were reduced, with levels decreasing to less than those for pancreatic cyst lesions. Yu et al[24] similarly used a microfluidic platform with high EpCAM+ CTC capture in the peripheral blood, however, the use of a single fluorescence channel for EpCAM and cytokeratin (CK) resulted in an inability to distinguish if the positive signal was coming from EpCAM, CK, or both. Although microfluidic platforms hold a lot of promise, the varied methodologies and enumeration criteria along with rapid advances in technology[25,26] still require validation with regard to balancing CTC isolation sensitivity with purity.[27]

The paucity of CTCs identified in the peripheral blood of patients with PC has been attributed to hepatic sequestration. Hematogenous dissemination via the portal venous circulation is supported further by the predominant location of gastrointestinal metastases, including PC, to the liver. CTCs in portal venous blood have been described via intraoperative acquisition in patients with colorectal liver metastases, in which portal venous blood CTC numbers were significantly higher compared with peripheral blood (median, 87 vs 1 CTC/7.5 mL, respectively).[13] Our findings of significantly higher levels of EUS-acquired/CellSearch-identified CTCs in nonmetastatic portal venous blood compared even with metastatic peripheral blood is consistent with the following hypotheses: (1) tumor cells are shed into the vasculature before the clinical/radiologic detection of metastases, and (2) CTCs from the primary tumor are being filtered during their transit through the hepatic portal circulation, resulting in fewer cells entering the systemic circulation. These points emphasize the importance of the blood collection site for CTC detection in PC curative-intent scenarios, and likely any tumor draining primarily via the portal venous system. Given the safety profile of EUS and no demonstrated complications with EUS-guided portal vein sampling, portal venous blood CTC enumeration may rapidly become adjunctive to current methods of PC risk stratification.

CTC enumeration, via EUS-guided portal venous blood sampling, has the potential to provide clinical utility with prognostic stratification and therapeutic decision making. In this study, we show the ability to use CTCs from portal venous blood, at the time of diagnosis, to determine the expression of tumor-suppressor genes, *p16/CDKN2A*, *TP53*, and *SMAD4*, which are inactivated most frequently in PCs, but any other protein of interest also could be assessed. Smad4, an intracellular component of the transforming growth factor-β signaling pathway, is inactivated in approximately 60% of PCs and is associated with a poor prognosis.[28,29] The shorter overall survival and increased risk of distant metastases has led researchers to suggest sparing patients the risk of pancreatic surgery, particularly in borderline resectable patients with concomitant *SMAD4* gene inactivation.[30] In 1 patient with stage IV metastatic PC, we identified increased smad4 protein expression in portal venous CTCs (1.6-fold increased relative to controls) (Figure 3*E*), which was confirmed by DNA sequencing and protein expression with IHC from a metastatic lymph node. The *p16/CDKN2A* gene encoding the protein p16 is inactivated in the majority of PCs via promoter methylation, missense mutations, or deletions

associated with loss of heterozygosity.[31,32] In the same patient, despite having a nonmutated *p16/CDKN2A*, we identified loss of p16 protein expression by IHC in the lymph node as well as similar expressions in portal venous CTCs and control cells. Although pathologic diagnoses of PCs frequently are made via EUS-guided FNA cytology examination, we show here that EUS-guided portal venous blood sampling, at the time of diagnosis and/or after neoadjuvant therapy, can enumerate CTCs and allow for molecular characterization of specific cancer-associated genes/proteins with the potential to provide guidance for preoperative risk stratification and/or selecting neo-adjuvant chemotherapy. The ability to isolate portal venous blood CTCs and complete tumor molecular profiling serially over time may provide significant information that will facilitate personalized clinical decision making.

In addition to the direct clinical benefits, EUS-guided portal venous blood sampling of extravasated primary tumor cells in the form of CTCs may provide select tissue specimens to further investigate metastatic tumor biology during a critical window period of hematogenous spread because emerging evidence suggests that occult metastatic seeding may occur before large primary tumor formation and even detectable invasive carcinoma.[33,34] CTCs now are recognized to represent a heterogeneous population of cells,[11,12] including apoptotic cells, cells undergoing epithelial-to-mesenchymal transition with loss of epithelial markers, epithelial cells, and cell clusters. There has been increasing evidence that cell clusters may be the precursor population responsible for distant organ metastatic seeding[35] and here we show that portal vein blood sampling can identify CTC clusters. CTCs already have been analyzed with next-generation sequencing,[36] and analyzing portal vein CTCs likely will shed new light on the biology of PBC metastases.[11]

We have shown that portal venous CTCs are far more common and higher in absolute numbers than peripheral blood CTCs. We also have shown the feasibility of obtaining portal venous CTCs noninvasively via EUS, as well as the capability to characterize these isolated cells molecularly. Future prospective studies will define the role of portal vein CTCs as prognostic and/or predictive biomarkers in the perioperative setting.

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.2015.08.050.

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Conflicts of interest
The authors disclose no conflicts.

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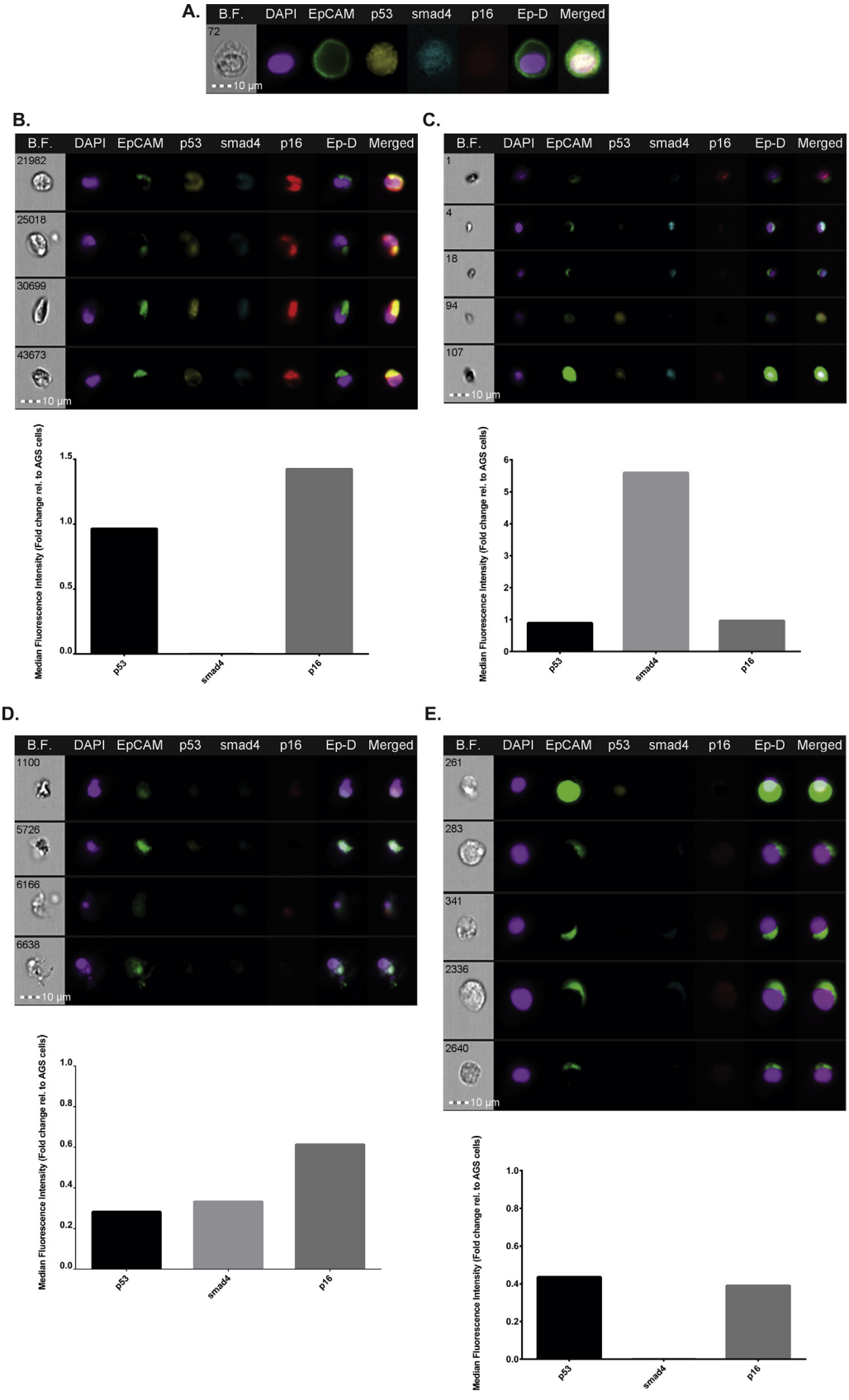
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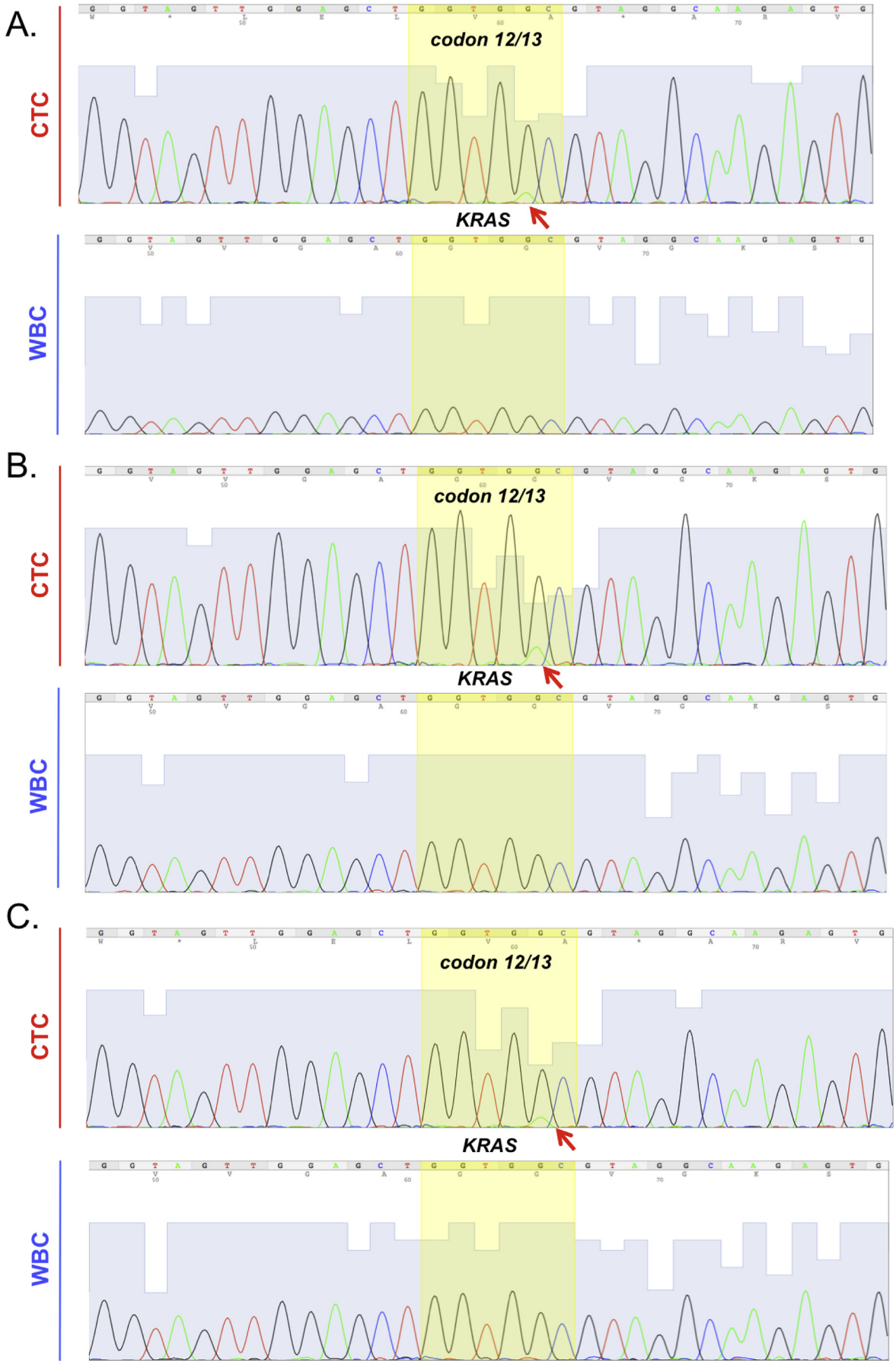
Supplementary Figure 1. Tumor-suppressor genes, *p16/CDKN2A*, *TP53*, and *SMAD4* protein expression in AGS control cells and portal venous CTCs. (*A*) Representative immunofluorescent staining of the proteins in individual AGS control cells. (*B–E*) Representative ImageStream immunofluorescence images of p53, smad4, and p16 proteins in CD45-depleted EpCAM+, DAPI+ portal venous CTCs and associated median intensity for immunofluorescence relative to AGS controls normalized to mean cell size (mean aspect ratio of the bright field) in individual patients with (*B*) stage IIB pancreatic cancer, (*C*) stage III pancreatic cancer, (*D*) stage IV pancreatic cancer, and (*E*) stage IV pancreatic cancer.


A.
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Supplementary Figure 2. Confirmation of *KRAS* mutations detected in portal venous CTC DNA. Whole-genome amplification followed by targeted Sanger sequencing chromatograms of *KRAS codons 12/13* detecting the presence of Gly13Asp (GGC>GAC) mutations in individual patients with (*A*) stage IIB pancreatic cancer, (*B*) stage IV pancreatic cancer, and (*C*) stage IV pancreatic cancer. Concurrent, paired portal vein–acquired white blood cell (WBC) fractions were used as sequencing controls for each patient with no evidence of *KRAS codon 12/13* mutations. *Yellow highlight* indicate codons 12 and 13 of *KRAS*. *Red arrows* indicate the sequencing location of the *KRAS* gene with Gly13Asp (GGC>GAC) mutation in portal vein CTCs.