Circulating Tumor Cells Found in Patients With Localized and Advanced Pancreatic Cancer

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Objectives: Isolation of circulating tumor cells (CTCs) holds the promise of diagnosing and molecular profiling cancers from a blood sample. Here, we test a simple new low-cost filtration device for CTC isolation in patients with pancreatic ductal adenocarcinoma (PDAC).

Methods: Peripheral blood samples drawn from healthy donors and PDAC patients were filtered using ScreenCell devices, designed to capture CTCs for cytologic and molecular analysis. Giemsa-stained specimens were evaluated by a pancreatic cytopathologist blinded to the histological diagnosis. Circulating tumor cell DNA was subjected to *KRAS* mutational analysis.

Results: Spiking experiments demonstrated a CTC capture efficiency as low as 2 cells/mL of blood. Circulating tumor cells were identified by either malignant cytology or presence of *KRAS* mutation in 73% of 11 patients ($P = 0.001$). Circulating tumor cells were identified in 3 of 4 patients with early (≤American Joint Committee on Cancer stage IIB) and in 5 of 7 patients with advanced (≥ American Joint Committee on Cancer stage III) PDAC. No CTCs were detected in blood from 9 health donors.

Conclusions: Circulating tumor cells can be found in most patients with PDAC of any stage, whether localized, locally advanced, or metastatic. The ability to capture, cytologically identify, and genetically analyze CTCs suggests a possible tool for the diagnosis and characterization of genetic alterations of PDAC.

Key Words: circulating tumor cells, pancreatic cancer, *KRAS* mutation

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Insufficient progress has been made in the understanding of the pathology, diagnosis, and therapy of pancreatic cancer.[1,2] Although the morbidity and mortality after pancreatic surgery has greatly decreased,[3] 80% of patients with pancreatic ductal adenocarcinoma (PDAC) are found to have nonresectable locally advanced or metastatic disease at the time of diagnosis.[4–6] There is a need for new biomarkers for earlier diagnosis and for stratification of patients eligible for resective surgery.

Circulating tumor cells (CTCs) are such a potential biomarker. Circulating tumor cells are rare cells in transit in the blood stream of patients with solid tumors, which are most likely shed from the primary tumor.[7,8] These may be living clones of the primary tumor with the potential to initiate metastasis in distant organs.[9] Circulating tumor cells have been reported in association with breast, prostate, and lung cancer and are being tested as a tool for both diagnosis and monitoring during chemotherapy.[8,10–12] Recent developments in technology have given rise to various CTC isolation platforms and protocols.[9,12,13] However, many of the techniques use costly, technically demanding systems based on antibodies to epithelial cell markers for cell selection and enrichment.[14–17]

An alternative to these complex techniques is the isolation by size of epithelial cells with ScreenCell (Paris, France) devices, a marker-independent, filtration-based method grounded on cell size and morphologic criteria. These are single-use units that capture the CTCs, which are larger than normal blood cells. Their key unit is a polycarbonate filter with micropores (diameter of 7.5 ± 0.36 μm, randomly distributed throughout the filter; 1×10^5 pores/cm^2), which allow normal blood components to pass through the filter but retain the much larger CTC.[18] After isolation, cells can be stained for cytologic evaluation using Giemsa stain or lysed for DNA extraction and molecular analysis with the ScreenCell MB kit. More than 90% of PDAC harbor somatic mutations in the *KRAS* proto-oncogene.[19,20] *KRAS* mutations can thus serve as a biomarker to verify the isolated CTC as malignant.

The aim of this prospective, proof-of-principle, exploratory study was to test with this application the CTC recovery for cytological diagnosis and genetic evaluation in patients with localized, locally advanced, or metastatic PDAC.

MATERIALS AND METHODS

Human Samples

Peripheral blood samples were drawn from 9 healthy controls and 11 patients with pancreatic cancer after obtaining informed consent in accord with the Massachusetts General Hospital institutional review board. Healthy donor blood was spiked by adding varying known quantities of PDAC cells to test the sensitivity of detection. The PDAC blood was drawn before surgery or palliative chemotherapy and processed through the ScreenCell devices within 3 hours.

TABLE 1. Molecular Analysis and Cytology Results of Spiking Experiments

	1000 cells/mL (n = 4)	100 cells/mL (n = 4)	10 cells/mL (n = 4)	2 cells/mL (n = 4)	Negative Control (n = 9)	PANC-1 Control (n = 4)
$KRAS^{G12D}$ mutation	100%	100%	100%	100%	0%	100%
Malignant cytology result	100%	100%	100%	50%	0%	100%

Spiking experiments (n = 16). Specified numbers of PANC-1 cells were added to 3 mL (cytology device) or 6 mL (molecular biology device) of normal blood: 1000 cells/mL (n = 4), 100 cells/mL (n = 4), 10 cells/mL (n = 4), and 2 cells/mL (n = 4) of healthy blood were tested for cytology results and *KRAS* mutations. Buffy coat and PANC-1 DNA was used as negative and positive controls, respectively.

Cytological Evaluation of CTC

For cytological detection of CTC, 3 mL of blood was filtered according to manufacturer's instructions using the ScreenCell Cyto kit. Briefly, the blood is incubated with the ScreenCell FC buffer for 8 minutes, transfered into the top of the filtration unit, and drawn through the filter by a vacuum tube. After completing the filtration, the filter is rinsed with 500 µL of phosphate buffered saline, dried on absorbent tissue, and stained with Giemsa (Protocol Haem 3; Fisher) and toluidine blue stain.

The stained filters were evaluated by an experienced pancreatic cytopathologist (M.B.P.) blinded to the histological diagnosis. Cells were categorized as "negative/non-diagnostic," "suspicious," or "malignant" based on cytomorphological features. Cells considered malignant demonstrated an epithelial phenotype with enlarged nuclei, coarse chromatin, and irregular nuclear membranes. Suspicious cells also demonstrated an epithelial phenotype, but their nuclear features were bland with round smooth nuclei and even chromatin.

Detection of *KRAS* Mutations

For genetic analysis of CTC, 6 mL of blood was filtered using the ScreenCell MB device according to manufacturer's instructions. DNA from cells captured on the filter was isolated using the Qiagen Purgene core kit. A polymerase chain reaction with a peptide nucleic acid clamp, which binds and blocks amplification of the wild-type *KRAS* sequence but allows for exponential amplification of mutant *KRAS*, was used to identify mutations in codon 12 and 13 as previously described.[21] Polymerase chain reaction products were electrophoresed on a 4% agarose gel, and the presence of *KRAS* mutations was verified by sequencing (Genewiz, Boston, Mass).

Statistical Analysis

Statistical evaluation was performed with Graph pad prism for categorical variables. Fisher exact test was performed in instances of 2 and 3 variables.

RESULTS

Cytologic and Genetic Detection of PDAC Cell Lines Spiked in Whole Blood

Normal blood to which PDAC cells were added (spiked) was used to determine the sensitivity of CTC detection and the correlation between cytological and *KRAS* mutational capture results. PANC-1 cells that are known to contain a $KRAS^{G12D}$ mutation were spiked into whole blood to final concentrations of 1000, 100, 10, and 2 cells/mL. Cytological evaluation (Table 1) revealed the PANC-1 cells in all samples (n = 6) spiked with 10 cell/mL and above, and was able to detect PANC-1 cells in 1 of 2 specimens spiked with 2 cells/mL (Fig. 1; Table 1). Using mutant *KRAS* as a molecular biomarker for the presence of CTC, the $KRAS^{G12D}$ mutation was detected in all spiked samples (n = 8), including at concentrations as low as 2 cells/mL.

Cytological and Genetic Characterization of CTC from PDAC Patients

Blood samples from 11 patients with biopsy-proven pancreatic cancer and 9 healthy controls were studied for the presence of CTC. On cytological analysis, 2 specimens (18%) had cells with malignant cytological features either as single large abnormal cells (80 µm; Fig. 2A,) or as clusters of smaller cells—microemboli (20 µm; Fig. 2B). Seven specimens (64%) were categorized as

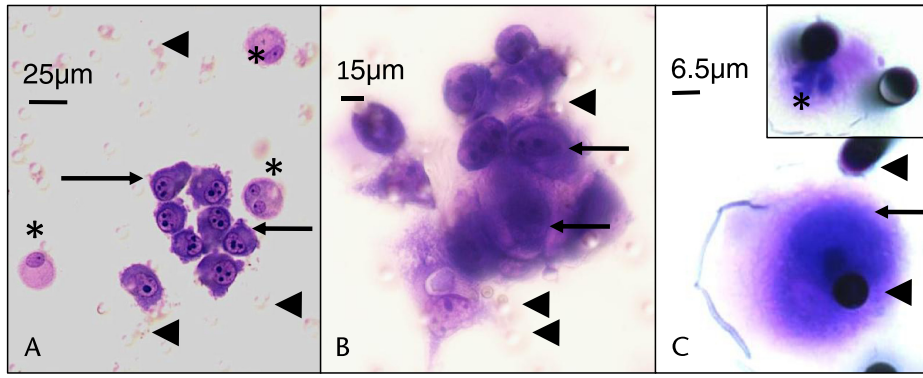

FIGURE 1. Cytological analysis of cells from spiking experiments. A and B, Representative images from a filter processed with healthy blood spiked with 1000 PANC-1 cells/mL and stained with toluidine blue. Tumor cells (long arrow), pores of the filter (arrowhead), and white blood cells (asterisk) are indicated. C, Representative image of Giemsa-stained cells from spiking experiment with 2 cells/mL. **Editor's note:** A color image accompanies the online version of this article.

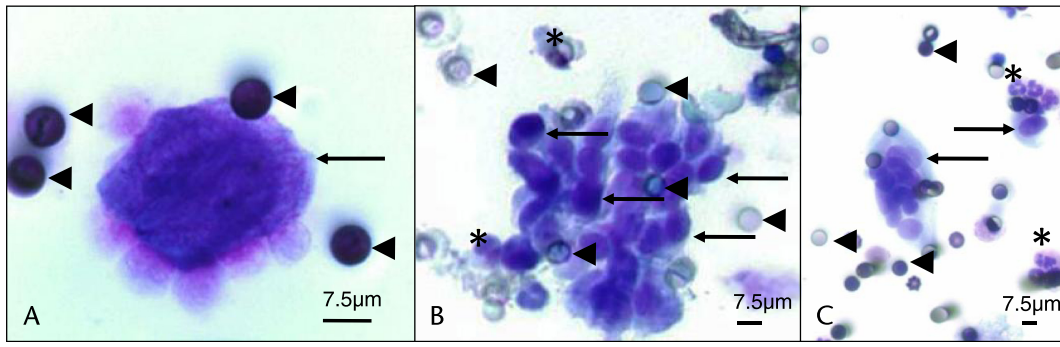

FIGURE 2. Isolated CTC in patients with PDAC. CTCs (arrows) were stained with standard Giemsa stain on filters (arrowheads indicate filter pores, asterisk indicates white blood cells) and evaluated by a cytopathologist (M.B.P.) blinded to the histological diagnosis. **Editor's note:** A color image accompanies the online version of this article.

suspicious (Fig. 2C). The remaining 2 (18%) were categorized as negative/non-diagnostic.

All 11 samples from PDAC patients were evaluated for *KRAS* mutations. In 8 (73%) of these 11 patients, *KRAS* mutations were identified. The 2 patient specimens diagnosed with malignant CTC by cytology were confirmed to have a *KRAS* mutation. *KRAS* mutations were also identified in 5 (71%) of the 7 patients with suspicious cytology and in 1 of the 2 samples that were considered negative/non-diagnostic (Fig. 3). Overall CTCs identified by either malignant cytology or presence of *KRAS* mutation as PDAC cells were found in 73% (8/11) of PDAC patients. In contrast, no malignant cells by either cytological or *KRAS* criteria were found in blood from 9 normal healthy control patients ($P = 0.001$, data not shown).

CTC and Stage of PDAC

In this pilot study, 36% of the patients had resectable cancers, which were American Joint Committee on Cancer (AJCC) stage IIB. The other 64% had locally advanced or metastatic disease; 27% had nonresectable locally advanced (AJCC stage III), and 37% had metastatic disease (AJCC stage IV).

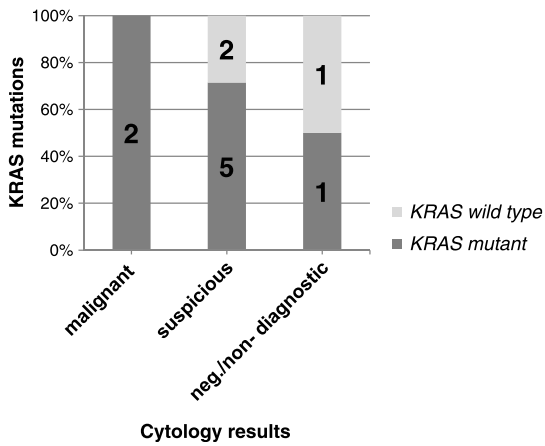

FIGURE 3. *KRAS* mutations and cytological appearance of CTC. Patient specimens captured on the cytology device were assessed by a cytopathologist in a blinded fashion as malignant, suspicious, or non-diagnostic. Parallel specimens isolated with the molecular biology device were subjected to *KRAS* mutational analysis. The numbers in each section indicate *KRAS* mutant specimens. **Editor's note:** A color image accompanies the online version of this article.

In this analysis, patients were considered CTC positive if they had either a *KRAS* mutation by molecular analysis or were identified to have malignant cells by cytology. Comparing CTC-negative and CTC-positive patients, the patient population demographics did not differ (Table 2). There was no difference in the rate of CTC detection between early-stage and advanced-stage disease ($P = 0.71$, Table 2); CTCs were identified in 75% (3/4) of patient with stage IIB, in 100% (3/3) with stage III, and in 50% (2/4) of patients with metastatic disease (stage IV). Prior neoadjuvant treatment did not seem to influence the detection of CTC as 83% (5/6) of the patients were found to have CTC after neoadjuvant treatment ($P = 0.36$).

DISCUSSION

In this study, we found CTC in the majority (73%) of 11 patients with pancreatic cancer with the use of the low-cost, technically simple ScreenCell filtration devices and a combination of both genetic and cytological criteria. This CTC detection rate is high when compared with previous studies on patients with locally advanced or metastatic PDAC using different antibody-dependent platforms, which had detection rates of 11% to 18%.[8,19] Cytology alone was able to identify CTC in 18% of cases in our study. Cytological characteristics of the CTCs were heterogeneous occurring as single large cells or clusters of smaller cells that aggregated to form microemboli (Fig. 2). When *KRAS* mutational analysis was added for cytologically suspicious specimens (Fig. 2C), the presence of mutations verified more CTC-positive

TABLE 2. Comparison of Demographic and Clinical Factors CTC Positive and Negative PDAC Patients

	CTC Positive (n = 8)	CTC Negative (n = 3)	*P*
Age, median, y	69	52	NS
Female sex	3	2	NS
PDAC patients	8	3	**0.001**
Control patients	0	9	
AJCC stage IIB	3	1	0.71
AJCC stage III	3	0	
AJCC stage VI	2	2	
Neoadjuvant chemoradiation	5	1	0.36

Comparison of patients with/without CTC. Demographic and clinical factors in patients with pancreatic cancer and healthy control patients.

NS, not significant.

patients (5 of 7). This suggests that the use of genetic biomarkers such as *KRAS* can be used to increase sensitivity of detection.

In this small pilot study, CTCs were identified with comparable frequency in both early- and late-stage disease; 3 of 4 patients with AJCC stage IIB were CTC positive. This may mean that the CTCs are disseminated early from the primary tumor. The identification of CTC in those patients eligible for curative surgery, and not only of those where palliative care is the only option, enhances their potential value as a biomarker. It is interesting that a recent study of intraductal papillary mucinous neoplasms, a known precursor lesion of a form of pancreatic adenocarcinoma, found circulating epithelial cells in the blood stream before the lesions became invasive.[22]

The benefits of the ScreenCell filtration devices are the simple speedy filtration technique that does not require expensive equipment or infrastructure. The filters can be read as a routine clinical cytological specimen (eg, a liquid biopsy). The isolation of DNA from the CTC is useful not only for diagnosis, but also might enable higher-order genomic studies. A study on CTC-RNA in patients with metastatic PDAC isolated with a different, more complex technique, the "herringbone chip" found the Wnt-signaling pathway activated in many CTCs as a potential reason for survival of these in transit cells.[23,24]

CONCLUSIONS

Using a low-cost filtration device, we found CTC in patients at all stages of PDAC. The CTCs were identified in 73% of patients with a combination of genetic biomarker and cytology. Validation and expansion of these findings holds the promise as a tool for new insights into the pathology of pancreatic adenocarcinoma.

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