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***Original Article***

**Possible novel non-invasive biomarker for chronic inflammation**

**induced chronic pancreatitis associated malignancy**

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**Abstract**

**Objectives:** Pancreatic malignancy is a major public health problem worldwide and recent reports indicated that pancreatic cancer will be second most common cause of cancer-related deaths by the end of 2021. The cause of increasing death rate is due to the nonexistence of detection tools to early diagnose, poor prognosis, resistance to chemotherapy and also lack in understanding the mechanism of PDAC pathogenesis. Circulating tumor cells (CTCs) play a major role in metastatic step of intravasation and presence of these cells are strong prognostic marker for the progression of pancreatic malignancy in chronic pancreatitis (CP). **Goal:** Identifying the novel CTCs in the chronic inflammation mediated experimental model for the progression of malignancy in CP. **Methods:** We have performed flow cytometer and immunofluorescence analyses were performed in the lymphoid and lung samples to detect CTCs in the chronic inflammation (Cerulin and Azoxymethane) induced CP mouse model. **Results:** We report that induced SOX9 positive cells were observed in the blood, lymph node and spleen samples of cerulein with azoximethane (AOM) treated mice compared to cerulein alone. Further, we provide evidence that early metastasis through the migration and homing of mega merged SOX9+ and PDX+ ductal stem cells (CTCs) in the lungs of cerulein with AOM treated mice. These identified CTCs in experimentally induced malignant pancreatitis may serve as a novel finding to identify a non-invasive biomarker that needs to be examined in the blood of human pancreatic cancer. **Conclusions:** Taken together, the presented data of identified mega merged SOX9+ and PDX+ ductal stem cells (CTCs) may serve a non-invasive biomarker for the early detection of pancreatic malignancy and metastasis.

**Keywords:** Biomarker, Circulating tumor cells (CTCs), Malignancy, Metastasis, Pancreatitis

**Introduction**

Pancreatic cancer that still stands among the most lethal cancers, with a low survival rate, and is a major cause of morbidity and mortality worldwide; there has been no change in the mortality rate over the past four decades.[1](#_ENREF_1), [2](#_ENREF_2) In 2019, there were over 56,700 new cases, with a similar number of deaths reported, making it the fourth most common cause of cancer mortality in the US because of its aggressive nature and treatment difficulty.[3](#_ENREF_3), [4](#_ENREF_4), [5](#_ENREF_5) The cause of the increasing death rate is due to the nonexistence of detection tools for early diagnosis, poor prognosis, and a lack in understanding the mechanism of the development of pancreatic cancer and metastasis. Oncogenic discovery efforts are currently focused on inhibiting the immune signaling checkpoint for targeted therapies and developing novel diagnostic strategies. [6](#_ENREF_6) The detection of characteristic tumor material in the blood is essential for establishing a noninvasive biomarker. Specific cancer-associated cancer tumor cells (CTCs) can be obtained from a routine blood draw with minimal risk and inconvenience to the patient compared to obtaining a fresh biopsy. [7](#_ENREF_7) In recent years, significant effort has been made to develop technologies that achieve specific and sensitive detection and capture of CTCs in several types of cancers. [8](#_ENREF_8), [9](#_ENREF_9) Notably, with the current available advances in technology, few CTCs are needed to detect and establish specific cancer-associated attractive alternatives to tumor tissue for biomarker analysis. CTCs will provide real-time information about the patient's current disease state. In the case of inflammation induce CP associated progression of pancreatic cancer and cancer metastasis, CTCs may be precursor cells from the pancreatic ductal system, which in turn fuse to antigen-presenting cells. [10](#_ENREF_10) In other cancers, these CTCs have been extensively studied, but in

cause of the increasing death rate is due to the nonexistence of detection tools for early diagnosis, poor prognosis, and a lack in understanding the mechanism of the development of pancreatic cancer and metastasis. Oncogenic discovery efforts are currently focused on inhibiting the immune signaling checkpoint for targeted therapies and developing novel diagnostic strategies. [6](#_ENREF_6) The detection of characteristic tumor

from the pancreas to the blood and lymph nodes, home to the lung and develop the characteristics of pancreatic cancer and metastasis in the lung. Our data also indicate why the pancreatic survival rate is low compared to other types of cancers. We observed that even before pancreatic tumors are visible, malignant pancreatitis metastasis starts to occur in the lung, and CTCs may move to other organs. These experimental findings from the murine model of CP that show the progression of characteristics features of malignancy provide a mechanistic understanding of the development of pancreatic cancer metastasis to the lung and establish a noninvasive CTC biomarker that needs further investigation to establish a similar CDC presence in human pancreatic cancer.

**Materials and Methods**

**Mice:** Specific pathogen-free Balb/c mice 8-10 weeks old were obtained from the Jackson laboratory (Bar Harbor, ME, USA), used as a wild type (WT) mice. The Institutional Animal Care and Use Committee (IACUC) approved the animal protocol in accordance with National Institute of Health (NIH) guidelines. All experimental mice were age (6–8 wk) and sex matched. Therefore, all the experiments performed are according to animal ethics rules and regulations.

**Developmental experimental model that develop pathological malignant characteristics in chronic pancreatitis (CP):** Experimental inflammation mediated development of pancreatic cancer characteristics using ceruline with azoxymethane (AOM) treated to mice. CP was induced by repetitive cerulein injections as described previously.[12](#_ENREF_12) AOM is a gene mutation agent was used to induce the chronic inflammation in the pancreas and develop pancreatic malignancy in CP. In brief, 6 weeks of Cerulein (Sigma-Aldrich, St. Louis, MO) was given by repetitive intraperitoneal injections as reported earlier (50 μg/kg, 6 hourly injections/day; 3 days/wk) along with 10 mg/kg AOM or saline. Mice were sacrificed 3 days after the last cerulein injection (**Fig.1A**), and blood, lymphoid organs and tissues were collected and processed for the analysis.

**Developmental experimental model that show**

material in the blood is essential for establishing a noninvasive biomarker. Specific cancer-associated material in the blood is essential for establishing a noninvasive biomarker. Specific cancer-associated cancer tumor cells (CTCs) can be obtained from a routine blood draw with minimal risk and inconvenience to the patient compared to obtaining a fresh biopsy. [7](#_ENREF_7) In recent years, significant effort has been made to develop technologies that achieve specific and sensitive detection and capture of CTCs in several types of cancers. [8](#_ENREF_8), [9](#_ENREF_9) Notably, with the current available advances in technology, few CTCs are needed to detect and establish specific cancer-associated attractive alternatives to tumor tissue for biomarker analysis. CTCs will provide real-time information about the patient's current disease state. In the case of inflammation induce CP associated progression of pancreatic cancer and cancer metastasis, CTCs may be precursor cells from the pancreatic ductal system, which in turn fuse to antigen-presenting cells. [10](#_ENREF_10) In other cancers, these CTCs have been extensively studied, but in the inflammation mediated pancreatic malignancy, their significance is still unknown.They play a major role in the metastatic step of intravasation, and the presence of these cells is a strong prognostic marker for pancreatic cancer patients. Earlier studies showed that in pancreatic ductal adenocarcinoma development, SOX9 plays a central role [11](#_ENREF_11) because SOX9 has been shown to be expressed in IPMNs in pancreatic malignancy. Therefore, identifying CTCs will be a unique biomarker for potential applications such as staging, prognosis and treatment options for pancreatic cancer. Recently, we developed a murine model of CP that show several characteristic features of pancreatic malignancy and mimics human PDAC characteristics shown in histological, biochemical, and molecular analyses, including ductal cell metaplasia, merger of pancreatic ducts, angiogenesis and the induction of pancreatic cancer-associated oncogenic proteins (KRAS, P53, SOX9, SMAD4) (manuscript under communication). In this report, we present evidence of the presence of few CTCs in the blood, spleen and lymph nodes in our inflammation-mediated murine model of CP that show the progression of pancreatic malignancy. We present evidence that SOX9-expressing pancreatic cancer ductal cells merge with CD11b-expressing macrophages via the PD1-PDL1 interaction, move

were mounted with nuclear staining DAPI mounting material. The images were captured using an Olympus BX51 microscope with appropriate filters, and photomicrographs are presented as original magnification ×400. Each mouse slide wasexamined for three to four random sections at ×400 magnification. There were six mice in each group.

**Statistical analysis.** The nonparametric Mann–Whitney *U*-test wasemployed for comparison of data between two groups, and Krustal–Wallis forcomparison of more than two groups. Parametric data were compared using *t* -tests or analysis of variance. Values are reported as mean ± S.D. *P*-values < 0.05 were considered statistically significant in InStat GraphPad.

**Results**

‘**Murine model of CP that develop malignant** **characteristics:** We established that a 6-week regimen of 3 doses of cerulein with azoxymethane (AOM)-treated mice resulted in the development of several characteristics of pancreatic cancer such as ductal cell metaplasia, merger of pancreatic ducts and formation of PanINs that mimicked the characteristics of pancreatic malignancy in humans, in particular to pancreatic adenocarcinoma (PDAC) (**Figure 1E**). Although several histologically characteristic features of pancreatic cancer were observed in our 6-week protocol regimen of CP, no visible pancreatic or lung tumors were observed in mice. It is interesting to note that these characteristics were not observed in mice treated with cerulein alone or saline and AOM-treated control mice (**Figure 1B-D**). The cerulein-treated mice showed most of the pancreatitis characteristics, including the accumulation of several inflammatory cells, acinar cell hypertrophy and ductal cell hyperplasia, but no ducts were merged compared to no histological changes in saline-treated mice. Similarly, we also observed airway epithelial cell hyperplasia similar to that observed in lung adenocarcinoma in the murine model of CP compared to the accumulation of some inflammatory cells and epithelial cell proliferation in cerulein-treated mice (**Figure 1H-I**). Normal lung pathology was observed in saline and

**pathological malignant characteristics in chronic pancreatitis (CP):** Experimental inflammation mediated development of pancreatic cancer characteristics using ceruline with azoxymethane (AOM) treated to mice. CP was induced by repetitive cerulein injections as described previously.[12](#_ENREF_12) AOM is a gene mutation agent was used to induce the chronic inflammation in the pancreas and develop pancreatic malignancy in CP. In brief, 6 weeks of Cerulein (Sigma-Aldrich, St. Louis, MO) was given by repetitive intraperitoneal injections as reported earlier (50 μg/kg, 6 hourly injections/day; 3 days/wk) along with 10 mg/kg AOM or saline. Mice were sacrificed 3 days after the last cerulein injection (**Fig.1A**), and blood, lymphoid organs and tissues were collected and processed for the analysis.

**Histopathological analysis:** Mice pancreatic

tissue specimens were fixed with 4% paraformaldehyde and embedded in paraffin using standard techniques. The paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin (H&E) to analyze the histopathological characteristics in tissue sections of experimental pancreatitis.

**Flow Cytometer Analysis:** Mouse blood, lymph node and spleen samples were collected from CP mouse model and processed for flow analysis as per the protocol reported earlier.[13](#_ENREF_13) The cells were stained with the following combination of antibodies along with a live/dead cell marker and different fluorochrome-labeled antibodies; anti-CD45, anti-CD11b, anti-PD1, anti-PDL1 (Bio legend), and anti-Sox9 (clone D8G8H) according to animal ethics rules and regulations.

**Immunofluorescence analysis:** Paraffin-coated mouse pancreatic tissue sections were deparaffinized, blocked with normal goat serum to reduce nonspecific binding, and incubated with anti-Sox9 (D8G8H) antibody (1:50; Cell Signaling) and anti-PDX1 antibody (658A5) (1:50; Cell Signaling) overnight followed by anti-mouse IgG- FITC and PE-labeled (Biolegend, San Diego, CA) secondary antibody. The immunostained sections

antibodies. Our analysis detected the homing of PDX1- and SOX9-positive cells in the lungs of cerulein- and AOM-treated mice compared to no such cell homing in the lungs of cerulein or saline -treated mice (**Figure 2D-F**). Pancreatic cancer ductal cells merged with leucocytes were detected (CD45+Sox9+CD11b+) in the blood, lymph nodes, spleen, and lung indicate inflammation mediated 

pancreatic cancer metastatic cells migration from the pancreas to lung. Notably, for the first time, our established model detected novel CD45+CD11b+SOX9+ mega-merged cells in the

AOM-treated mice (**Figure 1F-G**). Representative photomicrographs of the pancreas and lung sections are shown, n=6 mice/group.

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**SOX9+ pancreatic stem ductal cells are detected in the blood, lymph nodes, and spleen.** These findings prompted us to investigate the mechanism that is operational in the development of inflammation mediated pancreatic malignancy-associated lung fluorochrome-tagged leucocyte-specific anti-CD45, macrophage-specific anti-CD11b, cancer cell-specific anti-SOX9, and pancreatic duct-specific anti-PDX1 antibodies and analyzed them by flow cytometry. The analysis detected anti-SOX9+CD45+CD11b+ merged cells in the blood, lymph nodes and spleen (**Figure 2A-C**) of cerulein with AOM-treated mice compared to cerulein-treated mice.

**Pancreatic cancer associated metastasis is detected in the lungs of a murine model of CP.** Furthermore, we examined pancreatic cancer metastasis in the lungs of cerulein-treated mice compared to cerulein-treated mice. We present evidence of pulmonary adenocarcinoma-type epithelial cell hyperplasia in the lung bronchioles. We performed immunofluorescence staining of lung tissue sections by using leucocyte-specific CD45, macrophage-specific anti-CD11b, and pancreatic ductal cancer-expressing molecule anti-SOX9

(PanIN), ductal cell metaplasia (ADM) and intraductal papillary mucinous neoplasm (IPMN), etc. The rationale to examine these circulatory CTCs expressing these molecules is based on the fact that pancreatic cancer stem ductal cells express SOX9 [18](#_ENREF_18) as well as PDL1, [19](#_ENREF_19) and pancreas-accumulated macrophages express PD1. [20](#_ENREF_20) Our investigation indicates that PD1-expressing macrophage function and tumor-cell expression of PDL1 are critical in the formation of these mega-merged cells and, with the help of macrophage-specific chemoattractants present in the lung, contribute to its homing for cancer cells metastasis. These findings of mega-merged cells in experimental models will be novel prognostic noninvasive biomarkers that need to be established in human malignant pancreatitis. CTCs are also known as a “liquid biopsy” and are used as a surrogate biomarker in several cancers, including lung cancer. [21](#_ENREF_21) CTC analysis is a simple noninvasive method that causes less pain and discomfort than tissue biopsy.[22](#_ENREF_22) Earlier, CTCs were used for the diagnosis of metastasis and recurrence in lung cancer and were monitored via changes in CTCs before and after surgery or chemotherapy. CTCs have been proven to be helpful in improving the follow-up treatment regimen and thus enhance the survival rate and quality of life of patients. [23](#_ENREF_23)

Taken together, the data described here show that CTC detection using flow cytometer analysis is novel and useful for the early-stage detection of pancreatic malignancy and the further characterization of metastatic cells. Survival rate in pancreatic cancer is very low; therefore, our experimental findings are critical and need attention to be established as a novel noninvasive biomarker for human malignant pancreatitis. Even though, we detected very few CTCs in mice, but hope that based on these findings our efforts will obtain sufficiently similar CTCs in the blood of patients suffering from pancreatic cancer. More importantly, the CTC analysis is a prospective setting for molecular diagnosis in cases when tumors are even not very visible in CP patients.

**Author Contributions**

SUV performed all flow cytometer experiments and data analysis, and manuscript figure preparation. HKK and MM, generated animal model histolo-

blood and lymph nodes that home to the lungs of mice. These novel findings provide us with a tool to mice. These novel findings provide us with a tool to understand the mechanism operational in pancreatic cancer metastasis to other organs and its early detection as a novel diagnosis. Additionally, we also examined the mechanism that operates in the merger of these CD11b-Sox9+ cells. Macrophages express PD1, and pancreatic ductal cancer cells express PDL1 [14](#_ENREF_14); therefore, the PD-PDL1 interaction may be critical for the formation of these mega-merged metastatic cells. We present evidence that CD11b-SOX9+ cells express both PD1 and PDL1 (**Figure 2G**). The detection of mega-merged CTCs in our experimental model of PDAC is a novel finding and may lead to the first identified noninvasive biomarker, as well as the target cell for early development of pathological characteristic features of pancreatic cancer.

**Discussion:**

The etiology of pancreatic cancer remains largely elusive. Smoking and alcohol are often implicated as risk factors and are associated with an approximately three-fold increase in the risk of pancreatic cancer; less than 5% of cases of pancreatic cancer are thought to be related to increasing death rate is the late diagnosis in pancreatic cancer patients. [4](#_ENREF_4) The mechanistic understanding of chronic inflammation-induced pancreatic malignancy is not yet well understood, even though inflammatory cytokines and chemokines are implicated in genetically mutated experimental models and human pancreatic cancer. [16](#_ENREF_16) Many reports have indicated that pancreatic cancer is an extremely aggressive malignancy characterized by a high metastatic burden at the time of diagnosis. [17](#_ENREF_17) The events leading to metastatic spread in patients are largely unknown; therefore, we tested whether pancreatic cancer metastasis involves macrophages and ductal cell merger via PD1-PDL1 interaction and migration through blood and lymph nodes and homing into different organs with the help of cell-specific chemoattractants. Our analysis indeed detected anti-SOX9+CD45+CD11b+ merged cells in the blood, lymph nodes and spleen of our presented mouse model of CP that progress into the development of several pancreatic cancer features like pancreatic intraepithelial neoplasia

gical examination and data analysis. SK, technical help in processing the tissue samples and animal injection, AM, designs, supervised, and wrote the manuscript.

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**Competing Financial Interest.**

Authors declare no competing financial interest.

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